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EDITED BY

SIMON FLEXNER, M.D.

PEYTON ROUS, M.D.

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VOLUME FORTY-NINTH
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RELATION OF VARICELLA TO HERPES ZOSTER.

I. STATISTICAL OBSERVATIONS.

By T. M. RIVERS, M.D., AND L. A. ELDRIDGE, JR., M.D.

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(Received for publication, February 13, 1929.)

Varicella, herpes simplex, and herpes zoster are diseases characterized by vesicular eruptions in the skin. Macroscopically and microscopically the individual lesions (1-4) in these maladies are frequently very similar. In spite of the striking similarity exhibited by the individual lesions, the picture as a whole, *i.e.*, distribution of the rash and immune reactions, is as a rule characteristic for each disease.

It is now generally believed that varicella and varioloid are caused by different viruses. Such a remark at present is trite, but a hundred years ago the difference between the diseases was questioned by many observers, and certain workers (5), even 30 years after Jenner's report on vaccination, still thought that chicken-pox and smallpox were produced by the same infectious agent.

Herpes simplex is so frequently associated with other diseases, *e.g.*, pneumonia, meningitis, malaria, that the name of symptomatic herpes is often used in referring to it. Through the recent work of Grüter (6), Löwenstein (7), and others, it is now known that this type of herpes, occurring alone or in connection with other diseases, is caused by a specific virus. As soon as it was admitted to the virus group, a discussion arose regarding its relation to herpes zoster. From Cole and Kuttner's (8) review of the literature and from their work concerning the identity of the diseases, it seems unlikely that the majority of cases of herpes simplex and herpes zoster are caused by the same virus.

Herpes zoster, because of its frequent association with injury of nerves and spinal ganglia, for a long time (1861) was considered to be a manifestation of trophic disturbances in the skin attendant upon altered innervation. This conception, however, of the etiology was not applicable to all cases of zoster, and soon (1878) certain cases came to be looked upon as being caused by an infectious agent. In 1900, Head and Campbell (9) reviewed the literature and presented their work upon the subject. They concluded, because of the changes observed in the spinal ganglia and nerves, in the skin, and in the lymph nodes, that many of their cases of zoster were produced by a specific virus. Other cases, however, were thought to be caused not by a virus but by a mechanical or chemical irritation of spinal ganglia.

In 1909 Bókay (10) set forth a hypothesis, based on clinical observations, that some cases of zoster (herpes zoster varicellosa) are caused by the virus of chicken-pox and that such cases are capable of giving rise to varicella in exposed susceptible individuals. Following Bókay's report, numerous observations confirming or refuting his idea have been recorded. Most of the reports are similar to those made a hundred years ago in regard to the relation of chicken-pox to varioloid and smallpox and consist in recording the occurrence of varicella in individuals exposed to zoster and *vice versa* or in relating the coexistence of the two diseases in the same person or in individuals in the same family. Chicken-pox is a common disease and zoster is not rare (11), consequently reports of the nature above mentioned are of little value, inasmuch as coincidence alone doubtless accounts for the apparent relation of the two diseases in certain instances. There are reports, however, of a different nature that must be considered seriously. These will be discussed in the second paper of this series.

The real problem regarding the diseases under discussion is not whether the virus of herpes simplex under rare conditions can cause a disease indistinguishable from zoster, or whether the virus of varicella under the proper circumstances is capable of producing a malady similar to zoster and *vice versa*, but whether there are 3 distinct viruses, though they be very much alike in certain respects, each of which as a rule causing a distinct clinical picture, herpes simplex, herpes zoster, and varicella. If the same virus is instrumental in producing the majority of the cases of any 2 of the diseases, certain types of statistical data should reveal this fact. The purpose of the present paper is to present such data that seems to bear upon the identity of the causal agents of the majority of cases of herpes zoster and varicella.

Methods and Materials.

Herpes Zoster.—The records of cases of herpes zoster at the Bellevue and Vanderbilt clinics were examined and from them information concerning the monthly incidence and the age incidence of the disease was obtained. The selected clinics are large and their records undoubtedly are good indices of the prevalence of zoster at any given time throughout New York City.

Varicella.—The data concerning the monthly incidence of chicken-pox in New York City from Jan., 1914, through July, 1926, and from Aug., 1926, through Dec., 1926, were obtained respectively from the Monthly and the Weekly Bulletins of the Department of Health of the City of New York. The information regarding the age incidence of varicella was derived from the records of cases of chicken-pox observed at the Rockefeller Hospital, 1922–28.

TABLE I.
Tabulation of the Number of Cases of Varicella Reported Each Month for 13 Years in New York City.

Year	Jan.	Feb.	Mar.	Apr.	May	June	July	Aug.	Sept.	Oct.	Nov.	Dec.	Total
1914	1,121	1,156	1,222	1,217	1,451	1,104	319	85	56	315	574	1,112	9,732
1915	1,341	1,134	1,423	1,073	989	1,220	277	89	77	149	288	661	8,701
1916	956	874	1,439	1,623	1,747	1,331	430	70	44	303	325	631	9,779
1917	874	876	1,294	957	1,212	1,183	217	78	64	141	584	906	8,386
1918	785	598	559	441	542	363	175	95	56	136	174	272	3,776
1919	414	412	599	693	963	786	187	67	63	169	287	686	5,326
1920	726	492	536	467	629	680	227	80	74	230	485	908	5,534
1921	1,212	1,166	1,311	924	939	806	263	49	59	119	410	683	7,941
1922	841	656	663	540	673	490	131	48	67	207	475	947	5,738
1923	1,289	997	780	785	1,235	1,259	398	134	65	195	538	996	8,471
1924	1,155	1,018	1,200	1,000	857	767	230	108	83	318	636	858	8,260
1925	845	864	909	798	871	1,211	250	86	68	164	588	892	7,616
1926	1,091	874	645	456	542	731	343	111	57	241	650	963	6,704
Total.....	12,650	11,147	12,580	10,974	12,650	11,951	3,447	1,100	833	2,687	6,014	10,521	95,994
Mean 1914-26.	973	857	967	844	973	919	265	84	64	206	462	809	7,384
Mean 1922-26.	1,044	708	839	716	836	892	270	97	78	225	577	931	

TABLE II.

Tabulation of the Number of Cases of Herpes Zoster Observed Each Month for 13 Years at the Bellevue Clinic.

Year	Jan.	Feb.	Mar.	Apr.	May	June	July	Aug.	Sept.	Oct.	Nov.	Dec.	Male	Female
1914	2	2	1	5	1	3	2	3	1	2	1	2	14	11
1915	3	0	5	0	2	4	1	2	4	3	2	2	14	14
1916	0	1	2	2	2	0	3	1	1	1	2	4	11	8
1917	1	2	1	3	2	0	0	2	4	2	2	2	12	9
1918	6	0	2	0	2	4	2	1	3	2	2	0	15	9
1919	4	5	5	1	2	4	0	2	1	4	0	3	19	12
1920	2	3	3	2	3	3	0	4	3	3	2	3	19	12
1921	2	3	5	2	1	3	5	4	3	3	4	2	24	13
1922	8	5	2	3	7	2	4	2	4	3	7	1	35	13
1923	4	3	2	7	7	5	4	2	2	4	1	5	32	14
1924	2	2	3	3	4	4	3	2	3	6	4	3	30	9
1925	5	3	4	3	3	5	2	3	5	0	2	3	31	7
1926	4	5	0	2	5	3	4	6	4	0	1	4	24	14
Total.....	43	34	35	33	41	40	30	34	38	33	30	34	280	145
Mean 1914-26..	3.3	2.6	2.7	2.5	3.2	3.1	2.3	2.6	2.9	2.5	2.3	2.6		
Mean 1922-26..	4.6	3.6	2.2	3.6	5.2	3.8	3.4	3.0	3.6	2.6	3.0	3.2		

TABLE III.

Tabulation of the Number of Cases of Herpes Zoster Observed Each Month for 5 Years at the Vanderbilt Clinic.

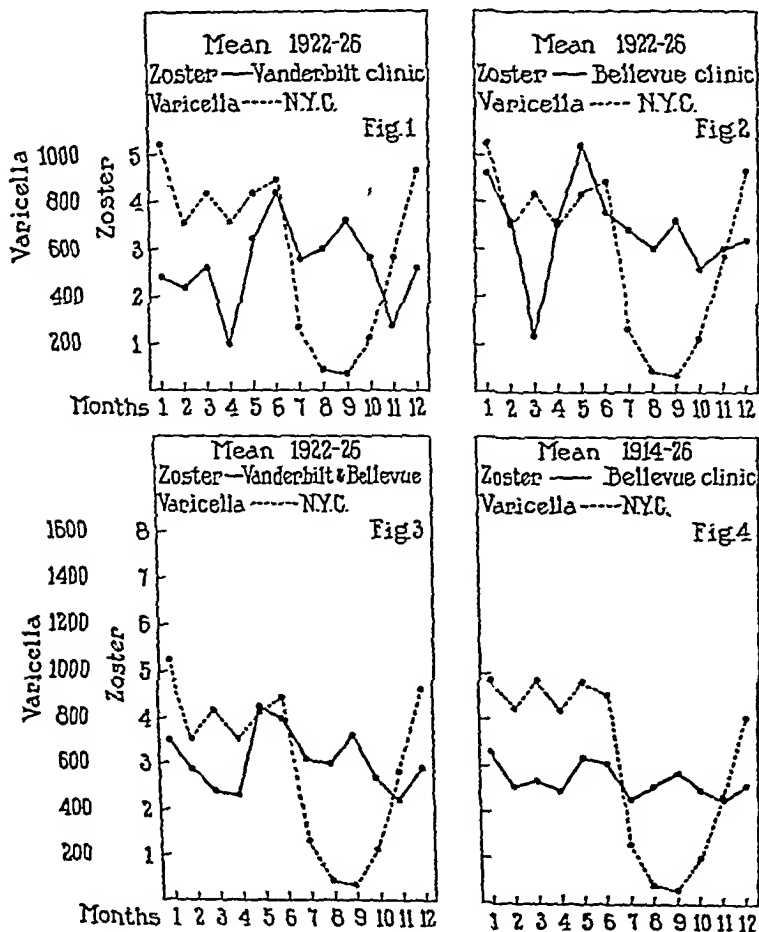
Year	Jan.	Feb.	Mar.	Apr.	May	June	July	Aug.	Sept.	Oct.	Nov.	Dec.
1922	3	2	3	0	3	5	1	4	1	1	3	2
1923	1	3	2	1	5	5	3	5	2	6	2	5
1924	4	2	2	0	2	5	5	5	5	1	0	0
1925	3	4	3	3	2	2	2	1	8	1	1	2
1926	1	0	3	1	4	4	3	0	2	5	1	4
Total...	12	11	13	5	16	21	14	15	18	14	7	13
Mean...	2.4	2.2	2.6	1	3.2	4.2	2.8	3.0	3.6	2.8	1.4	2.6

RESULTS.

Monthly Incidence of Zoster and Varicella.

In New York City during the 13 years, 1914-26, 95,994 cases of chicken-pox were reported. In Table I these cases have been set down

in a manner to indicate the incidence of the disease for each month during the 13 years. At the Bellevue clinic, 425 cases of herpes zoster were observed during the same period of 13 years, and 159



FIGS. 1-4. Curves showing the monthly incidence of varicella in New York City and herpes zoster in the Vanderbilt and Bellevue clinics.

cases were registered at the Vanderbilt clinic during the 5 years, 1922-26. In Tables II and III these cases of zoster have been arranged according to their monthly incidence. The significant features of the

information in Tables I to III have been graphically portrayed in Figs. 1 to 4.

Examination of the tables and figures dealing with the monthly incidence of varicella and zoster reveals the fact that a remarkably constant seasonal variation occurs in the number of cases of chicken-pox reported, while such variation in the prevalence of zoster is not very obvious. In fact, when the mean of a large number of cases over

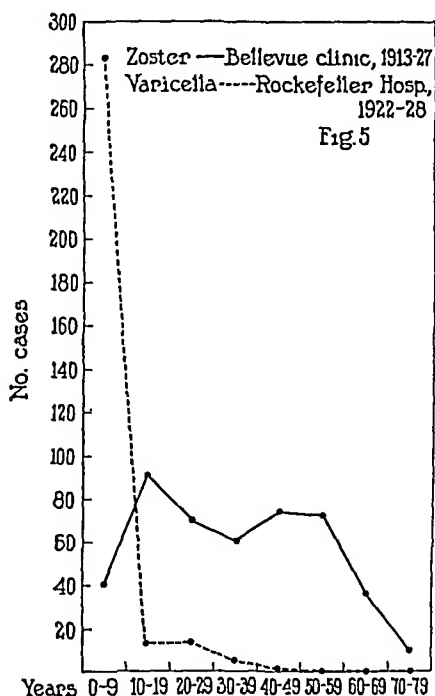


FIG. 5. Distribution curves illustrating the age incidence of varicella and herpes zoster.

a period of many years is plotted (Fig. 4), the curve indicating the monthly incidence of zoster approaches a straight line.

Age Incidence of Zoster and Varicella.

In Table IV the age of 457 cases of zoster and of 318 cases of chicken-pox has been indicated. From the data in Table IV, distribution curves (Fig. 5) for the 2 diseases have been plotted. From the facts presented in Table IV and Fig. 5, it is quite evident that varicella occurs for the most part in individuals under 10 years of age, while zoster most frequently appears in the 2d, 3d, 4th, 5th and 6th decades of life. Obviously there is a striking difference in the age incidence of these diseases.

DISCUSSION.

The difference in the age incidence of herpes zoster and varicella has been well recognized for a long time (11) and facts concerning it are again presented in this paper merely for emphasis. That there is a constant seasonal variation in the prevalence of chicken-pox is also well known, and certain authors (9, 12) have recorded observations concerning the monthly incidence of zoster. Up to the present time,

however, very few attempts have been made to compare the number of cases of these diseases occurring simultaneously in the same locality. Perutz (13) made such a comparison in 2 districts of Vienna for the years 1923-25 and found that the curves of monthly incidence of the 2 diseases did not parallel each other. Moreover, Cantor (14) reports that on Christmas Island,¹ Straits Settlement, where excellent vital

TABLE IV.

A Tabulation of Varicella and Herpes Zoster Cases Showing the Difference in the Age Incidence of the 2 Diseases.

Herpes Zoster, Bellevue Clinic, 1913-27, 457 Cases.

Varicella, Rockefeller Hospital, 1922-28, 318 Cases.

Age	Zoster	Varicella	Age	Zoster	Varicella	Age	Zoster	Varicella	Age	Zoster	Varicella
0	1	18	19	13	2	38	8	1	57	6	0
1	1	32	20	7	5	39	3	0	58	8	0
2	1	27	21	6	0	40	12	1	59	6	0
3	4	48	22	6	1	41	7	0	60	9	0
4	2	38	23	6	1	42	10	0	61	2	0
5	6	45	24	4	0	43	5	0	62	4	0
6	6	39	25	8	4	44	6	0	63	0	0
7	7	17	26	13	0	45	7	0	64	5	0
8	7	11	27	10	3	46	8	0	65	6	0
9	6	8	28	4	0	47	5	0	66	4	0
10	11	4	29	7	1	48	5	0	67	1	0
11	11	2	30	4	0	49	10	0	68	3	0
12	9	2	31	5	0	50	12	0	69	3	0
13	13	1	32	9	1	51	6	0	70	1	0
14	10	2	33	5	2	52	11	0	71	0	0
15	3	2	34	7	0	53	7	0	72	2	0
16	9	0	35	8	0	54	5	0	73	2	0
17	6	0	36	6	0	55	5	0	74	2	0
18	6	0	37	5	0	56	6	0	75	3	0

statistics have been kept for 20 years, chicken-pox is unknown, while the usual amount of herpes zoster is observed. The statistical observations recorded in the present paper are further evidence in favor

¹ This island was uninhabited before 1888. The population, 500 to 1500, consists of Europeans, Indians, Malays, and Chinese. Both sexes and all ages are represented.

of the idea that the majority of cases of herpes zoster is not caused by the virus of varicella.

SUMMARY.

Varicella most frequently occurs in individuals under 10 years of age, while zoster as a rule is observed in persons beyond that age. The number of cases of varicella exhibits a markedly constant seasonal variation. The variations in the prevalence of herpes zoster are not regular and do not parallel those of varicella.

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RELATION OF VARICELLA TO HERPES ZOSTER.

II. CLINICAL AND EXPERIMENTAL OBSERVATIONS.

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In the preceding paper (1) the problem relating to the identity of the causal agents of varicella and herpes zoster was stated. The mere occurrence of varicella in an individual following exposure to herpes zoster might easily be explained upon coincidence. Certain other observations, however, dealing with complement fixation work, inoculation experiments, and protection tests conducted with convalescent sera deserve serious consideration.

Netter (2) and Netter and Urbain (3), de Lange (4), and others, because of their results with complement fixation tests, contend that the etiological agents of zoster and varicella are quite frequently identical. Lauda and Silberstern (5), however, were unable to confirm these observations. Kundratitz (6) and Lipschütz and Kundratitz (7) inoculated 28 children under 5 years of age with material from herpes zoster patients in the hope of immunizing them against chicken-pox. In 17 of the children a local reaction, characterized by papules and vesicles, was observed at the point of inoculation. The children were subsequently found to be immune to varicella. These writers also claim that injections of convalescent herpes zoster serum will protect exposed children against varicella. Lauda and Stöhr (8) inoculated 54 children with material from 17 cases of herpes zoster, and in none of them did they observe a local reaction at the site of the inoculation. Three of the inoculated children and 3 other uninoculated infants who came in contact with some of the zoster patients developed typical chicken-pox 2 or 3 weeks after exposure. These workers were unable to demonstrate that convalescent zoster serum protects against varicella and conclude from the results of their work that the majority of cases of zoster are not caused by the virus of chicken-pox.

Chicken-pox is a highly contagious disease, but for a long time doubt existed in regard to its inoculability. A great deal of this doubt arose from the fact that most of the experimental inoculations were made during epidemics of varicella and exposures to the disease under natural conditions could not absolutely be eliminated. Nevertheless, in spite of these difficulties, it has been shown (9) that

varicella is inoculable. Successful experiments, however, are not as constant as one might suppose when considering the extremely contagious nature of the disease. Evidence is accumulating in favor of the idea that zoster also is caused by a virus, but the question as to its inoculability in humans is still moot.

If it were possible without difficulty to produce varicella and zoster in animals, the relation of the two diseases would be settled quickly. It is very doubtful if the virus that causes the majority of cases of zoster has been propagated in experimental animals. With one exception this is also true in regard to varicella. Certain kinds of monkeys (*Cercopithecus sabæus* and *Cercopithecus lalandi*) are susceptible to the virus of chicken-pox. Even in these animals the results are irregular and the evidence of infection consists of the occurrence of acidophilic nuclear inclusions in affected cells of inoculated testicles. These inclusions, however, do not occur in controls and their appearance is regularly prevented by convalescent varicella serum (10, 11). In view of the regularity with which convalescent varicella serum prevents these inclusions in testicles inoculated with chicken-pox virus, it seemed likely that information regarding the identity of varicella and zoster might be obtained by conducting neutralization tests with varicella virus and convalescent zoster serum. It is with observations concerning these experiments that the present paper deals.

Methods and Materials.

Monkeys Employed.—Young male green monkeys (*Cercopithecus sabæus*) were used. Animals in which spermatogenesis had been established were discarded.

Neutralization Tests.—Emulsified papules and vesicles collected from varicella patients, usually within the first 72 hours of the disease, were used as virus. Non-immune serum was secured from chicken-pox patients during the first 72 hours of disease. Immune varicella serum was obtained from convalescent patients 14 to 22 days after the appearance of the rash. Immune zoster serum was collected from convalescent patients 23 to 28 days after the appearance of the eruption.

The papules and vesicles were excised under aseptic conditions and emulsified by grinding in a mortar moistened with Locke's solution. Sand was not used. The emulsified material was taken up in 0.5 to 2.0 cc. of Locke's solution and portions of it were mixed as desired with equal amounts of non-immune serum, immune varicella serum, or immune herpes zoster serum. Measured amounts (0.25 cc.) of the mixtures were then injected into the testicles of monkeys. More than 45 minutes never elapsed between the collection of the varicella virus and its injection into the animals.

Removal and Examination of Testicles.—It was previously shown (10, 11) that nuclear inclusions are present in the testicles of green monkeys on the 5th and 6th days after inoculation with chicken-pox. Consequently, in the experiments reported at the present time, the monkeys were castrated¹ on the 5th or 6th day following inoculation. The testicles removed for histological studies were fixed in Zenker's fluid, sectioned, and stained with eosin and methylene blue. A careful search for eosin-staining nuclear inclusions was made in numerous sections of each testicle. Details concerning the tinctorial reactions of the inclusions are given by Tyzzer (12), Lipschütz (13), and Goodpasture (14).

Results.—Testicles in which nuclear inclusions were found were considered infected with varicella virus. If no inclusions were found, infection was considered not to have occurred. In this type of work, positive results are naturally much more significant than are negative ones.

EXPERIMENTAL.

In the study of the relation of varicella to herpes zoster 4 neutralization experiments were performed.² A detailed account of each follows.

Experiment I.

The first experiment was conducted in order to determine whether the serum of an adult, who had had chicken-pox in childhood, would neutralize varicella virus.

Serum was collected from J., 35 years of age, who had chicken-pox when 5 years old. Non-immune serum was obtained from W. R. during the first 48 hours after the appearance of the varicella rash. Virus in the form of 4 varicella vesicles was secured from B. The serum to be tested, from J., was mixed with an equal amount of virus, and then 0.25 cc. of the mixture was injected into each testicle of Monkey 1. Non-immune serum, W. R., was also mixed with virus, and 0.25 cc. of the mixture was injected into each testicle of Monkey 2. 5 days later the testicles were removed and examined in the usual manner for the presence of acidophilic nuclear inclusions. They were found in the testicles of both monkeys.

¹ All operative procedures were conducted under anesthesia.

² Because of the scarcity of green monkeys, control tests with convalescent varicella serum were at times omitted. In fact, it is not essential to use this control with each experiment, inasmuch as convalescent varicella serum has never failed to prevent the appearance of inclusions in testicles inoculated with varicella virus (11).

The results of the above experiment indicated to us that the serum from an individual who had had varicella many years previously did not contain sufficient antibodies to prevent the appearance of inclusions in testicles inoculated with chicken-pox virus. Consequently it was deemed safe in this work to use convalescent zoster serum obtained from individuals who a number of years earlier in life had passed through an attack of varicella.

Experiment II.

In this experiment an attempt was made to ascertain whether the serum from a case of so called idiopathic herpes zoster was capable of neutralizing varicella virus.

C. S., male, white, 16 years old. No history of having had chicken-pox or zoster previously. No exposure recently to either disease. Patient seen on the first day of the zoster eruption which was distributed over the right sacral and iliac regions; serum collected. Lesions healed slowly. Serum again collected 23 days after onset of the disease.

In the neutralization tests, 4 monkeys were used. Virus, 9 lesions, was collected from 3 varicella patients (G. K., G. M., H. B.). Non-immune serum (W. R.), immune serum (M. W.), and serum collected from C. S. on the 1st and 23d days after the appearance of the zoster eruption were employed. Virus and non-immune serum were injected into Monkey 3; virus and convalescent varicella serum into Monkey 4; virus and early zoster serum into Monkey 5; virus and convalescent zoster serum into Monkey 6. 6 days after the inoculations, the testicles were removed, fixed, sectioned, and stained. Upon examination no inclusions were found in any of the sections.

In view of the negative results in all the monkeys, the experiment was repeated. At this time, however, only non-immune serum (W. R.) and convalescent zoster serum (C. S.) were used. The virus, 6 varicella vesicles, was obtained from A. M. and W. McK. Virus and non-immune serum was injected into Monkey 7; virus and convalescent zoster serum into Monkey 8. 5 days later the testicles were removed and examined in the usual manner. In sections from the testicles of Monkey 7 numerous nuclear inclusions were found, while none were seen in those from Monkey 8.

From the results of this experiment one might conclude that the convalescent zoster serum neutralized the varicella virus. Monkeys, however, display a certain amount of irregularity in their susceptibility to chicken-pox virus. Consequently one set of negative findings is not highly significant.

Experiment III.

The following test was made to determine whether the serum from a patient who developed herpes zoster following the administration of salvarsan would neutralize chicken-pox virus.

M. R., female, colored, age 36. No history of previous attacks of chicken-pox or herpes zoster. No known recent exposure to either disease. ++++ Wassermann. On Jan. 13, 1927, at the Vanderbilt clinic, the patient received her first intravenous injection of salvarsan. 24 hours later a burning sensation was felt over the left shoulder, and 48 hours following the treatment blisters were seen over the left side of neck and left shoulder. Patient was seen at the Rockefeller Hospital for the first time on Jan. 21 and was found to have typical herpes zoster. The lesions healed slowly. Convalescent serum was collected on Feb. 11.

Virus, 4 fresh vesicles, was collected from a varicella patient on the 3d day of eruption. Virus and non-immune serum (W. R.) were injected into Monkey 9; convalescent zoster serum (M. R.) and virus were inoculated into Monkey 10. 5 days after the injections the testicles were removed and examined for the presence of acidophilic nuclear inclusion. In the testicles of both animals many were found.

In this experiment it seems that the convalescent zoster serum did not neutralize the chicken-pox virus.

Experiment IV.

The experiment described below is the most important one in the series. In it was used serum from a convalescent zoster patient who had had varicella $7\frac{1}{2}$ years previously and from whom a sister appeared to have contracted chicken-pox through exposure to her during the attack of zoster.

M. B., female, white, age 8. No history of previous attacks of zoster. Had chicken-pox when 6 months old. No recent exposure to varicella or zoster. Attends a public school. On Oct. 24, 1926, the patient developed blisters over the right side of the body. She was not quarantined and came in contact with relatives, classmates, and playmates. On Nov. 1, she was seen at the Vanderbilt clinic and referred to the Rockefeller Hospital. At that time the patient presented the picture of typical herpes zoster. The eruption was on the right side of the body over an area supplied by the 9th and 10th thoracic nerves. The child was not admitted to the hospital, but was seen from time to time in the clinic. Dr. E. visited the home on several occasions and a nurse observed the children in the patient's class at school. The lesions on the child healed slowly, and convalescent serum was obtained on Nov. 20, 26 days after the appearance of the eruption.

V. B., age 3, a sister who had never had varicella or zoster was constantly exposed to the patient. On Nov. 13, 19 days after the appearance of zoster in M. B., a chicken-pox eruption was observed on V. B. The child was admitted to the Rockefeller Hospital and after passing through a typical attack of varicella was discharged on Nov. 27. Just prior to discharge convalescent serum was obtained.

The parents said that V. B. had not been exposed to varicella, yet she developed the disease 19 days after exposure to herpes zoster in her sister. This, then, is an excellent example of chicken-pox developing in a child after exposure to herpes zoster. Many such examples have been reported and have been adduced as evidence of the identity of the two diseases. Upon pursuing the matter further, however, it was found that M. B.'s class at school consisted of 33 children, 21 of whom had never had varicella. All of them were exposed to M. B., yet not a case of chicken-pox developed as a result of the exposure.

Four monkeys were used in the neutralization tests. The virus consisted of 12 vesicles removed from 3 varicella patients (J. R., W. R., V. D.). Non-immune serum (W. R.), chicken-pox convalescent serum (M. W.), convalescent serum from M. B., and convalescent serum from V. B. were respectively mixed with equal amounts of virus. 0.25 cc. of the mixtures were then injected into the testicles of monkeys; virus and non-immune serum into Monkey 11, virus and convalescent varicella serum into Monkey 12, virus and convalescent serum from M. B. into Monkey 13, virus and convalescent serum from V. B. into Monkey 14. 5 days after the injections the testicles were removed and examined in the usual way for the presence of nuclear inclusions. Sections from Monkeys 11 and 13 showed numerous inclusions, while those from Monkeys 12 and 14 revealed none.

The results of the experiment indicate that the non-immune serum and the serum from the child (M. B.) convalescent after zoster did not neutralize varicella virus, while convalescent varicella serum of extraneous derivation and convalescent serum from the sister (V. B.) who had just had chicken-pox did inhibit its action. In view of these findings, together with the failure of varicella to develop in any of the classmates of the child (M. B.) who had zoster, one is justified in concluding that the relation of chicken-pox to zoster in the two sisters, V. B. and M. B., was apparent rather than real.

A summary of the experiments dealing with the neutralization of varicella virus by convalescent herpes zoster serum is given in Table I. An analysis of the results reveals that, under the conditions of the tests, sera from individuals who had had varicella $7\frac{1}{2}$ to 30 years previously did not neutralize varicella virus. This fact does not imply that these individuals were susceptible to chicken-pox and that their sera did not possess a certain amount of neutralizing power for varicella

virus. The experiments also indicate that 2 of 3 convalescent herpes zoster sera did not neutralize varicella virus while in each of 2 in-

TABLE I.
Summary of Neutralization Experiments.

Experiment	Monkey	Inoculum	Results
1	1	Varicella virus + non-immune serum	+
	2	" " + serum from adult who had varicella 30 years previously	+
2	3	" " + non-immune serum	-
	4	" " + convalescent varicella serum	-
	5	" " + serum from patient early in course of herpes zoster	-
	6	" " + serum from patient convalescent from herpes zoster	-
2 Repeat	7	" " + non-immune serum	+
	8	" " + convalescent herpes zoster serum	-
3	9	" " + non-immune serum	+
	10	" " + convalescent herpes zoster serum	+
4	11	" " + non-immune serum	+
	12	" " + convalescent varicella serum	-
	13	" " + convalescent herpes zoster serum from an individual (M. B.) who had had varicella 7½ years previously	+
	14	" " + convalescent varicella serum (V. B.)	-

Monkeys inoculated in testicles.

+ indicates infection by virus, determined by presence of nuclear inclusions in sections of inoculated testicles.

- indicates no infection by virus, determined by absence of nuclear inclusions in sections of inoculated testicles.

stances³ convalescent chicken-pox serum did neutralize it. An adequate explanation of why varicella virus appeared to be neutralized by one of the convalescent zoster sera is lacking. It is certainly not

³ Convalescent varicella serum regularly prevents the occurrence of nuclear inclusions in monkeys' testicles inoculated with varicella virus (11).

safe to conclude, even though the experiment was repeated, that the herpes zoster in that case was caused by varicella virus. From the experiments reported in this paper and those previously recorded in regard to varicella in monkeys (10, 11), one is justified in concluding that herpes zoster is not always produced by varicella virus. Such is the case even in instances where clinical observations might lead one to suspect that the causal agents of the two diseases are identical.

DISCUSSION.

Many investigators believe that certain cases of zoster are caused by a specific virus. Instances, however, of "recurring zoster," of zoster following mechanical or drug injuries to nerves, and of zoster occurring in patients with tabes, tuberculosis of the spine, and cord tumors have led numerous observers to consider that this type of the disease is due not to an infectious agent, but to trophic changes in the skin incident to nerve injury. Regardless of the conditions under which herpes zoster occurs, the character of the lesions has induced a number of workers familiar with virus diseases to entertain the idea that it is caused by some kind of virus.

The question as to whether zoster is caused by one or by several viruses naturally arises. The pathological changes observed in zoster lesions are strikingly similar to those seen in herpes simplex and varicella. In fact, lesions from these three diseases cannot be differentially diagnosed one from another by means of the microscope. As a group, however, they can be separated from other skin lesions occurring in man. This is made possible by the occurrence of acidophilic bodies in the nuclei of affected cells. From records of work dealing with the identity of herpes simplex and herpes zoster (15) it seems unlikely that the etiological agents concerned in the majority of the cases of these two diseases are identical.

It now remains to consider the relation of zoster to chicken-pox. The studies presented in the preceding paper (1), the experiments recorded in the present one, and the fact that an attack of herpes zoster as a rule does not protect against varicella and *vice versa* (16, 17, 18, 22) seem to indicate that the majority of the cases of the two diseases is not caused by the same virus. There is no good reason, however, why one should say that the virus of chicken-pox cannot under

certain conditions produce localized lesions clinically indistinguishable from herpes zoster. It probably does, but only rarely.

The conditions under which the virus of varicella causes a disease clinically similar to herpes zoster are probably dependent upon the rôle injury or irritation plays in the localization of many viruses. The viruses of measles (19) and varicella (20) localize in areas of irritated skin as is evidenced by a marked increase in the number of lesions appearing in such areas. Therefore, in hospitals where large numbers of tuberculous children and syphilitic infants undergoing treatment with arsenicals are cared for, a few cases of clinical zoster may occur during an epidemic of varicella. In all probability, under these conditions, some if not all of the so called zoster is caused by chicken-pox virus. Consequently it would be better to diagnose such cases as varicella with an abnormal localization of the rash.

It is not likely that trophic changes incident to nerve injury alone or to drug poisoning alone, *e.g.*, with arsenic, are capable of producing herpes zoster. If such were the case, the disease should be seen more often under these conditions. The fact that viruses tend to localize in irritated tissues may also account for the relation that has been observed to exist between certain cases of herpes zoster and nerve injury due to chemical, physical, or disease-producing agents. Whether the virus localizes primarily in ganglia and subsequently travels by way of nerves to the skin or whether the localization is only in the skin, the site being determined by circulatory and other changes incident to the injury of nerves supplying the region (21), is not definitely known. There is no reason why the primary localization of the virus may not occur in either place. The presence or absence of pain in zoster may in some instances be dependent upon the place at which the virus localizes.

Since in the majority of cases of herpes zoster the disease is caused neither by the virus of herpes simplex nor by the virus of varicella, is there any evidence at present that a special virus is concerned in its production? There is no direct proof that such a virus exists, yet the indirect evidence is suggestive. In the first place, the pathological picture presented by zoster lesions is one that has not been shown to occur in the absence of some kind of virus. Although zoster is not, as a rule, highly contagious, it breeds true when occur-

ring epidemically, *i.e.*, zoster gives rise to zoster (15, 22). Finally, in the majority of instances one attack of zoster confers an immunity against a second attack of the disease but not against an infection with herpes simplex and varicella viruses.

SUMMARY.

Experiments and clinical observations dealing with the identity of the viruses of varicella and herpes zoster were presented. The results indicate that the etiological agents concerned with these two diseases are in the majority of instances not identical.

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IMMUNOLOGIC REACTIONS WITH TOBACCO MOSAIC VIRUS.

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Ever since Mayer (1886) demonstrated the infectious nature of tobacco mosaic disease, investigations have been carried out to determine the true nature of the etiologic agent of this malady. Many of the objections to the parasitic theory based on the resistance of the infective agent to chemicals can be met with the argument that the usual methods for the determination of these reactions in the case of known microorganisms are crude when applied to the study of filterable viruses (Ford, 1927). Studies on the purification of virus have been made which may overcome some of the difficulties (McKinney 1927; Vinson and Petre 1929; and Brewer, Kraybill and Gardner 1929). The apparently high resistance to heat exhibited by virus is no greater than that shown by bacterial spores (Duggar and Armstrong 1923). Spores of *B. subtilis* show an increased resistance to heat when tested in vegetable infusions (Williams 1928).

The properties of tobacco virus are frequently likened to those of enzymes. An apparent similarity between them may be due to the fact that they have both been studied in tissue extracts and in an impure state. Falk (1924) maintains that the properties attributed to enzymes are in reality chiefly those of the medium in which they are tested. Allard remarked fourteen years ago that: "It may at least be said that the theory of a parasitic origin for the disease more consistently accounts for all the facts at hand than any enzymic conception yet evolved. It seems not only needless but illogical to abandon a simple, direct explanation for one which leads to complexity of thought and yet fails to correlate all the facts at hand." (Allard 1915).

Various investigators have proposed different hypotheses to explain the origin of tobacco mosaic disease. Some of them express the opinion that the malady is due to purely physiologic response to unfavorable environmental conditions (Egiz 1912; Hunger 1902, 1905 a, b; and Sturgis 1899). Hunger maintains that a phytotoxin is generated spontaneously in the plant which upon transmission to normal plants proves capable of stimulating them also to the production of the phytotoxin. Beijerinck attributes the disease to a "contagium vivum fluidum" (1898), while other workers think the excessive accumulation of oxidizing enzymes is responsible for the morbid condition of the plant (Heintzel 1900, Woods 1899,

1902, and Chapman 1913). Iwanowski, although unable to cultivate a microorganism from affected plants, considers the disease as bacterial in origin (1903). Allard (1916) expresses the opinion that the causal agent is an ultramicroscopic parasite. Several investigators have described microorganisms which they believe to be closely associated with the disease (Palm 1922; Jones 1926 a, b; Link, Jones, and Taliaferro 1926; Jones 1928; and Eckerson 1926). One of these supporters of the parasitic theory of tobacco mosaic disease claims that the foreign cell inclusions, characteristically present in tobacco mosaic tissue are the etiologic factor (Palm 1922).

Olitsky's claim of having cultivated the infective agent of tobacco mosaic disease¹ *in vitro* in the absence of living host cells (1924) is as yet uncorroborated (Mulvania 1925; Purdy 1926; Goldsworthy 1926; and Smith 1928).

Mulvania (1926) injected from 1 to 3 cc. of virus-sap in one ear of a rabbit and 20 minutes later tried to recover the virus by drawing a sample of blood from the uninoculated ear. The virus was not recoverable. He also showed that when normal, fresh, rabbit blood is mixed with virus extract and allowed to stand for 24 hours, the infectivity of the virus is not destroyed but under the same conditions, a filtrate of virus extract is rendered non-infectious. No attempt to produce an antiserum to virus-sap was reported by Mulvania.

Until the recent work of Dvorak (1927), on a virus of potato mosaic disease, no one had undertaken an investigation of the antigenic properties of virus-sap. Dvorak produced antisera in rabbits to sap from healthy potato plants and sap from potato affected with mosaic disease.² Precipitin reactions showed that the two antisera had precipitins in common, but that they both exhibited a higher titer for the homologous than for the heterologous antigen. The explanation offered for these results is that the virus had in some way altered the antigenic property of the globulins present in the healthy potato plant.

A study of the antigenic properties of virus-sap of tobacco mosaic should throw light upon the true nature of the virus. Therefore, the experiments described in this paper, reported previously in preliminary form (Purdy 1928), were undertaken.

Method.

Preparation of Antigens.—As a source of antigens young tobacco plants of a Turkish variety were selected because of a low nicotine-content, which would minimize any toxic effect the alkaloid might have on the rabbits used for immunization. Leaves were collected from healthy tobacco plants and from plants

¹ The host plant in this case was tomato.

² Although the filterability of the virus of potato mosaic disease has never been proved, it is nevertheless regarded as belonging to the class of filterable viruses.

that had been infected with mosaic disease for from 10 days to 6 weeks. The particular tobacco virus used was that of Johnson's common field mosaic disease.³ The diseased and healthy leaves were kept in two separate lots and prepared in vessels, sterilized each time after use to avoid any chance of contaminating the normal sap with virus-sap. Three medium-sized leaves were ground to a pulp in a mortar with 8.5 cc. of saline solution (0.85 per cent). The extract was then centrifugalized for about 5 minutes at a moderate speed and the supernatant fluid was used for injection. At first, the antigens were preserved with 0.5 per cent carbolic acid solution and stored in an ice-chest during the intervals between injections, but later fresh antigen was prepared for each injection to eliminate

TABLE I.
Hyperimmunization of Rabbits.

Rabbit No.	Antigen	No. of injections	Total amount of antigen injected	Period of hyper-immunization	No. of days after last injection, rabbit was bled
1	N*	6	23.0	Feb. 10-Mar. 6	6
2	N	5	16.5	Apr. 10-Apr. 23	10
3	N	6	19.0	Apr. 10-May 11	0†
4	V*	6	21.0	Feb. 10-Mar. 6	6
5	V	5	16.5	Apr. 10-Apr. 23	10
6	V	13	22.8	Apr. 10-June 7	14
7	V	7	9.2	July 25-Aug. 22	0†

* N = Normal sap.

V = Virus-sap.

† Rabbit died before bleeding.

the possibility of the proteins in the extracted sap becoming denatured upon standing.

Immunization of Rabbits.—Each rabbit received from 5 to 13 injections of antigen in the marginal ear-vein, the amount injected at one time varying from 0.5 to 5 cc. Since the rabbits showed toxic reactions when as much as 5 cc. were injected at one time, it was not deemed advisable to use a larger amount of sap extract. The usual interval between injections was 3 to 4 days. Before the fifth and subsequent injections, the rabbit was desensitized intravenously first with 0.5 cc. of antigen, otherwise severe anaphylactic symptoms were evinced. Four rabbits were injected with virus-sap and 3 with normal sap. Table I contains more details of the procedure followed in the injection of individual rabbits.

³ The original source of the virus was mosaic material supplied by Doctor James Johnson several years ago and subsequently kept free from contamination by other strains of virus at the Boyce Thompson Institute.

Collection of Serum.—In general, samples of serum were drawn from an ear-vein for control purposes, prior to the first injection of the antigen. Thereafter, frequent samples of serum were taken from an ear-vein during the course of injections for use in various tests. From 6 to 14 days after the last injection, the animals were etherized and bled to death aseptically from the carotid artery. The antiserum was allowed to separate from the clot overnight and was pipetted off the following morning, inactivated for one-half hour at 56°C. (water-bath) and stored in sterile tubes in an ice-chest.

Testing of Serum for Antibodies.

Preliminary alexin-fixation experiments were carried out in order to determine whether or not antibodies to virus and normal sap had been produced in the injected rabbits. It was found that in the presence of the homologous antigen, a given amount of serum from the injected animals would fix alexin completely, whereas the same amount of antigen and normal rabbit serum gave no fixation, that is, complete hemolysis occurred on adding sensitized sheep cells. The usual control tubes for antigen, hemolytic system, serum, and cells were included in the tests. All showed that the experiments were properly controlled.

The findings of these preliminary tests indicated that antibodies to virus and normal sap had been produced in the injected rabbits. Alexin-fixation experiments were then planned for the titration of both antigens with antisera to virus-sap and antisera to normal sap in order to ascertain whether or not the antisera contained alexin-fixing antibodies in common.

Alexin-Fixation Experiments.

The antisera and normal sera were heated for one-half hour at 56°C. (water-bath) just prior to use. Constant amounts (0.05 cc.) of undiluted serum were placed in Wassermann tubes. Fresh guinea-pig serum was then added of a dilution that would contain 2 units of alexin in each 0.1 cc. The antigens were prepared as for injection in the rabbits and were diluted further with saline solution (0.85 per cent). Decreasing amounts of antigen-dilution were pipetted into a series of tubes. The volume of liquid in each tube was then brought up to 0.25 cc. with saline solution. The entire mixture was shaken and placed in a water-bath for one hour at 37°C. At the end of the hour of incubation, sheep cells, previously sensitized with rabbit sensitizer, were added in 0.2 cc. amounts and the tubes were returned to the water-bath for 15 minutes. In some of the experiments, the readings were made immediately after removal from the water-bath; in other experiments the readings were made after the tubes had stood in the ice-chest overnight and the cells had settled out by gravitation. In general, it was found that a satisfactory and quicker method of obtaining the readings was to cool the

tubes under running water immediately after removal from the water-bath, and then centrifugalize them at a low speed for a few moments to throw down any non-hemolyzed cells present. In most of the tests, Madsen's scale was employed for determining the percentage hemolysis occurring in any of the tubes. Using the contents of tubes showing complete hemolysis as the stock solution, dilutions with saline solution were made showing 10 to 90 per cent hemolysis, by increments of 10 per cent.

Three separate experiments were carried out in which 3 different antiviral and 3 different antinormal sera were tested individually for their ability to bind alexin in the presence of virus and normal sap. The results of these experiments showed the smallest amount of antigen used that would react with 0.05 cc. of undiluted antiserum to fix a given amount of alexin. For the sake of discussion, these minimal amounts wherever available have been brought together in Table II. The amount of antigen used is expressed in the equivalent of undiluted sap to facilitate a comparison of the results.

Analysis of the Findings.—In all of 3 experiments (Table II) both antisera fix alexin in the presence of either antigen.

In all 5 titrations (Table II) antiviral sera show a higher titer for the homologous than for the heterologous antigen.

In all 3 experiments (Table II) antiviral sera possess a higher titer for virus than the antinormal sera for the same antigen.

In 3 out of 5 titrations (Table II, Experiments I, II, and IIIa) the antinormal sera exhibit a higher titer for the homologous antigen than for the heterologous antigen. Complete results were not obtained in the other 2 titrations (Table II, Experiment IIIb and c).

In 2 out of 5 titrations (Table II, Experiments I, and IIIa) the antinormal sera have a higher titer for normal sap than the corresponding antiviral sera for the same antigen; in one instance the titer of the antinormal and antiviral sera for normal sap is the same (Table II, Experiment II); in the other 2 titrations, the incomplete results would indicate that the antinormal sera have a lower titer for normal sap than the corresponding antiviral sera (Table II, Experiment IIIb, c).

Discussion.—In interpreting the results of the alexin-fixation experiments summarized in Table II, two possibilities should be taken into consideration—the differences that may exist in both the titer of the

antisera and the concentration of the antigens. These two factors influence the results, but if properly accounted for, they do not invali-

TABLE II.
Minimal Amounts of Antigen Required for Alexin-fixation.

Experiment No.	Undiluted serum (56°C.)		Undiluted antigen	Results	
				Hemolysis	Fixation
I	cc.	cc.			
	AV† 4	.006 V	—	Complete	
	AV 4	.02 N*	Partial	—	
	AN 1	.02 V*	Partial	—	
II	AN 1	.002 N	—	Complete	
	AV 4	.0015 V	—	Complete	
	AV 4	.008 N	—	Complete	
	AN 1	.01 V	—	Complete	
IIIa	AN 1	.008 N	—	Complete	
	AV 4	.001 V	—	Complete	
	AV 4	.03 N	—	Complete	
	AN 1	.03 V	—	Complete	
IIIb	AN 1	.02 N	—	Complete	
	AV 6	.0006 V	—	Complete	
	AV 6	.1 N	—	Complete	
	AN 3	.05 V	—	Complete	
IIIc	AN 3	.02 N*	75%	—	
	AV 5	.0006 V	—	Complete	
	AV 5	.05 N	—	Complete	
	AN 2	.1 V	—	Complete	
	AN 2	.02 N*	85%	—	

* Minimal amount of antigen was not determined.

† AV = Antiserum to virus-sap.

AN = Antiserum to normal sap.

V = Virus-sap.

N = Normal sap.

date the conclusions that may be drawn from these results. The following discussion will demonstrate this point.

Since antiviral serum fixes alexin in the presence of normal sap, it is fair to assume that antigenic substances in the normal sap were also

present in virus-sap used for the production of the antiviral serum. Likewise, since antinormal serum binds alexin in the presence of virus-sap, the reaction may be attributed to antigenic substances in virus-sap that were also present in normal sap. Without further knowledge of the antigenic nature of normal and virus-sap, the results obtained from these experiments may be examined to advantage on the assumption that the two antigens, virus and normal sap, are identical in composition. It is then possible to compare the titer of the antisera and the concentration of the antigens from the results of a single experiment in which antiviral and antinormal sera are titrated with the same preparations of homologous and heterologous antigens. The normal antigen and antinormal serum should be used as the basis for comparing the titer of the antisera and the concentration of the antigens, since the assumption that virus-sap is identical to normal sap in composition remains to be proved.

For example, in Experiment I (Table II) comparing the reaction of the 2 antiviral and antinormal sera to normal sap, the antinormal serum is the higher titered one. By further comparison of the reactions of antinormal serum with virus and normal sap, the former antigen appears to be less than one-tenth the concentration of the latter. By a similar comparison of the titrations in Experiment II (Table II), both antisera have the same titer, but virus-sap appears to be a somewhat weaker antigen than normal sap.

In all 5 sets of titrations, antiviral sera exhibit a higher titer for the homologous than for the heterologous antigen. On the other hand, the incomplete results of Experiment IIIb (Table II) indicate that the antinormal serum may manifest a higher titer for the heterologous than for the homologous antigen, depending in this instance upon the concentration of the antigens. Also, the antinormal sera may show a lower titer for the homologous antigen than that of the corresponding antiviral sera (Table II, Experiment IIIb, c) because of the higher titer of the latter. These results demonstrate the importance of titrating the 2 antisera with both antigens. Without the information thereby gained relative to the concentration of antigen and titer of antiserum, no dependable conclusions can be drawn from the results of a titration.

A careful study of the results obtained from these experiments makes

it evident that differences in the titer of the antisera or concentration of the antigens, identical in composition, are sufficient to explain the reactions of antinormal sera with both antigens, but not to account for the reactions of antiviral sera with virus and normal sap. For instance, in Experiment I (Table II) the antiviral serum is weaker in titer for normal sap than the corresponding antinormal serum, and the normal sap seems to be more highly concentrated than the virus-sap, from a comparison of the reactions of antinormal serum with both antigens. Under these conditions and with antigens of identical composition, the antiviral serum would be expected to show a higher titer for normal than for virus-sap, but in reality the reverse is true and antiviral serum exhibits a higher titer for virus than for normal sap. The only adequate explanation for such a result is that the 2 antigens differ in composition. Virus-sap apparently contains some antigenic substance or substances either not found in normal sap, or only in small amounts. It is possible that some foreign antigenic substance is present in virus-sap not found in normal sap, or that some of the normal antigens present in healthy tobacco sap have been altered by mosaic disease, thereby changing their antigenic properties.

The 2 antisera were now tested for the presence of precipitins.

Precipitin Experiments.

Four precipitin tests were set up, using equal parts of undiluted antiserum and antigen-dilution, varying from 1:10 to 1:100,000. The antigens were prepared as for injection in rabbits but it was found necessary to centrifugalize the extracts at high speed for 15 minutes to obtain sufficiently clear antigens. The supernatant fluid was further diluted with saline solution (0.85 per cent). The serum was first pipetted into precipitin tubes and the antigen-dilution was slowly "layered" on top of the serum. After incubation for 1 hour at 37°C. (water-bath), the tubes were examined for the presence of a ring of precipitate, formed at the region of contact between serum and antigen. The contents of the tubes were then thoroughly shaken and placed in an ice-chest overnight. On the following morning a record was made of the precipitate formed in the tubes.

Analysis of the Findings.—No precipitate was formed in a dilution of antigen exceeding 1:100.

A precipitate formed in the case of both antisera in the presence of either antigen.

A very slight precipitate formed in a mixture of normal sap and normal serum when the antigen was undiluted.

The virus-sap produced a heavier, more flocculent precipitate than the normal sap with both antinormal and antiviral serum. A greater amount of precipitate was obtained in a mixture of antiviral serum and virus-sap than in the case of antinormal serum and virus-sap.

Discussion.—The fact that the antigens failed to give a precipitate in a dilution exceeding 1:100, may be due to the fact that the antigen is prepared by the extraction of whole sap high in water-content. The antigens referred to as “undiluted” are in reality considerably diluted.

The heavier precipitate characteristically formed in a mixture of antiviral serum and virus-sap strongly suggests the presence of a specific precipitin for virus-sap in the antiviral serum. In comparing the reactions of the 2 antisera with their homologous and heterologous antigens, the same objections raised to a comparison of the results in the alexin-fixation experiments should be taken into consideration. In these tests, also, the titer of the 2 antisera and the concentration of the antigens may vary. It was concluded, therefore, in the face of these difficulties that the presence of a specific precipitin for virus-sap in antiviral serum could be more readily demonstrated by precipitin-absorption experiments.

Precipitin-Absorption Tests.

After a number of preliminary tests, it was found that the most satisfactory method for the absorption of the precipitins from the serum was as follows:

The antigens were freshly prepared as for the precipitin experiments and used in a dilution of 1 part of extracted sap to 4 parts of saline solution. The antisera were absorbed by adding 1 cc. of antigen-dilution to 2.5 cc. of inactivated serum and incubating for 1 hour at 37°C. (water-bath). The precipitate formed was then removed by centrifugalization and 1 cc. of antigen-dilution was added to the clear supernatant fluid. This process was repeated until none of the tubes showed any precipitate upon removal from the water-bath. The tubes were then set in the ice-chest overnight to allow ample opportunity for a precipitate to form. If the tubes contained no precipitate on the following morning the absorption of the serum was considered complete. The absorbed serum was then tested with both antigens for the presence of precipitins, as described in the precipitin experiments. During the process of absorption, several precipitin tests were carried out with the partially absorbed sera.

Two different antiviral sera and 1 antinormal serum were used in the 3 absorption tests. Two portions each of antiviral and antinormal serum were absorbed separately with both normal and virus-sap. Control tubes of antisera were included, treated in a similar manner to the sera absorbed except that saline was added each time in the place of antigen. Other appropriate control tubes were also used.

Analysis of the Findings.—All of the precipitins to normal sap in both antisera were completely absorbed by either antigen.

After complete absorption of antiviral serum with normal sap, a heavy precipitate was formed upon the subsequent addition of virus-sap.

Discussion.—During the process of precipitin-absorption, the antinormal serum was usually absorbed completely by the first addition of antigen while the largest amount of antigen used in these tests was required by the antiviral serum for complete absorption with virus-sap. For uniformity of method, the same quantity of antigen was used in every case for the absorption of the 4 different lots of serum in a single experiment. Consequently, it was necessary to set up complete precipitin tests at various stages during the process of absorption, otherwise the results would be incomplete, for the final precipitin tests showed that some of the absorbed sera were so highly diluted that no reaction occurred in the corresponding control tubes of unabsorbed serum.

The point of most interest in these experiments has been fully demonstrated by this method of precipitin-absorption, namely, that after complete absorption of an antiviral serum with normal sap, the absorbed serum will still give a heavy precipitate with virus-sap. As stated in the discussion of the results of the alexin-fixation experiments, the specific reaction may be due either to a foreign antigenic substance present in the virus-sap, or to the formation during the course of mosaic disease of an altered plant protein.

As a logical sequence to the precipitin-absorption tests, it was decided to repeat the experiments and instead of using tobacco sap for the final precipitin tests to substitute sap from healthy and mosaic diseased hosts of the virus other than tobacco.

Precipitin Tests with Tobacco Virus from Other Hosts.—Tomato, pepper, and petunia plants, common hosts of tobacco mosaic virus, were inoculated with the

TABLE III.

Precipitin-absorption Tests with Virus from Various Hosts of Tobacco Mosaic Virus.

Tube No.	Serum	Antigen used for absorption	Undiluted absorbed serum	Antigen dilution 1:10	Results	
					After incubation for 1 hr. at 37°C. (water bath)	After standing in ice-chest overnight*
			cc.	cc.		
1	AV†	N tobacco	.2	.2N tobacco	No ring	0
2	"	" "	.2	.2V tobacco	Ring	+++
3	"	" "	.2	.2N tomato	No ring	0
4	"	" "	.2	.2V tomato	Ring	+++
5	"	" "	.2	.2N pepper	No ring	0
6	"	" "	.2	.2V pepper	Heavy ring	++++
7	"	" "	.2	.2N petunia	No ring	0
8	"	" "	.2	.2V petunia	Very faint ring	++
9	"	" "	.2	.2 saline	No ring	0
10	"	" "	.2 saline	.2N tobacco	" "	0
11	"	" "	" "	.2V tobacco	" "	0
12	"	" "	" "	.2N tomato	" "	0
13	"	" "	" "	.2V tomato	" "	0
14	"	" "	" "	.2N pepper	" "	0
15	"	" "	" "	.2V pepper	" "	0
16	"	" "	" "	.2N petunia	" "	0
17	"	" "	" "	.2V petunia	" "	0
18	"	Saline	Unabsorbed serum .2	.2N tobacco	No ring	++
19	"	"	"	.2N tomato	Ring	+
20	"	"	"	.2N pepper	No ring	+
21	"	"	"	.2N petunia	" "	±
22	"	"	"	.2V tobacco	" "	++
23	"	"	"	.2V tomato	Heavy ring	+++
24	"	"	"	.2V pepper	Ring	++++
25	"	"	"	.2V petunia	Ring	++
26	"	"	"	.2 saline	No ring	0

* The following symbols are used:

0 = no precipitate.

± = very slight precipitate.

+ = slight precipitate.

++ = moderate precipitate.

+++ = heavy precipitate.

++++ = very heavy precipitate.

† AV = antiserum to virus-sap.

AN = antiserum to normal sap.

V = virus-sap.

N = normal sap.

strain of virus used throughout these experiments. The identical procedure used above in the precipitin-absorption tests was followed. Four sets of experiments were carried out. The results obtained from one of these tests are recorded in Table III. A parallel series of tubes with the corresponding unabsorbed serum was included in these experiments.

Analysis of the Findings.—When antiserum to virus-sap of tobacco was completely absorbed with normal tobacco sap (Table III):

(a) No precipitate was formed upon adding normal sap of tobacco, tomato, pepper, or petunia plants.

(b) A heavy precipitate was formed upon the addition of virus-sap of tobacco, tomato, pepper, and petunia plants.

Unabsorbed antiserum to virus-sap of tobacco produced some precipitate when mixed with the normal sap of tobacco, tomato, pepper, and petunia plants.

Discussion.—Since the 4 different host plants, tobacco, tomato, pepper, and petunia used in these experiments are all members of the same family, the *Solanaceae*, the occurrence of precipitins in antiserum to virus-sap of tobacco common to the 4 plants is to be expected. The presence of antigenic substances in tobacco common to all of these different plants still leaves the possibility unaltered that the antigenic substance in virus-sap of tobacco, tomato, pepper, and petunia specific for antiserum to tobacco virus-sap, may be plant protein altered by disease. If the specific antigenic substance is foreign virus protein, for example, its antigenic property is apparently not altered by multiplication in different hosts.

In the hope of determining the presence of an antibody, specific for virus itself, a series of experiments were undertaken to test the power of inactivation of the antiserum to virus-sap on virus.

Inactivation Experiments with Virus-Sap and Antiserum.

Known amounts of antiserum were pipetted into tubes and varying amounts of freshly extracted virus-sap, diluted with saline solution (0.85 per cent), were added to the serum. The volume of liquid in each tube was made constant by the addition of saline solution. In 4 out of the 6 experiments carried out, the mixture of serum and virus-sap was held at room temperature for several hours; in the other 2 cases the mixture was kept for 1 hour at 37°C. (water-bath). Two different antisera to virus-sap, 2 to normal sap, and several samples of normal rabbit and guinea-pig serum were tested.

After the serum had remained in contact with the virus-sap for a given period, the contents of each tube were shaken thoroughly, poured into a dish and used for inoculation into young Turkish tobacco plants.

The inoculation of the plants was accomplished by scarifying 3 leaves on each plant with a sterile needle, dropping on the inoculum with a capillary pipette, and rubbing it in with a sterile cork. Every precaution was taken throughout the

TABLE IV.

Summary of Results of Inactivation of Virus-sap with Antisera and Normal Serum.

Serum used for inactivation of virus	No. of individual mixtures of serum and virus-sap tested	No. of plants inoculated	No. of plants		Per cent of healthy plants
			Diseased	Healthy	
Active AV*	5	23	3†	20	86
(56°C.) AV	8	34	8‡	26	76
(56°C.) AV + alexin	7	32	3§	29	90
Total	20	89	14	75	88
Active AN	2	8	7	1	12
(56°C.) AN	1	6	3	3	50
(56°C.) AN + alexin	1	6	4	2	33
Total	4	20	14	6	30
Active normal	3	13	13	0	0
(56°C.) normal	1	6	6	0	0
(56°C.) normal + alexin	2	10	8	2	20
Total	6	29	27	2	6
Saline	6	26	26	0	0

* AV = antiserum to virus-sap.

AN = antiserum to normal sap.

† In 3 of 5 tests, complete inactivation occurred.

‡ In only 3 of 8 tests, complete inactivation occurred.

§ In 6 of 7 tests, complete inactivation took place. In the seventh instance, the corresponding amount of active serum also failed to produce complete inactivation.

entire procedure to avoid accidental infection of the plants. Records of the occurrence of mosaic were made as the disease developed, for a period of 4 weeks, when the plants were discarded.

Control tests were included in which the virus-sap was placed in contact with antiserum to normal sap, normal rabbit and guinea-pig serum, and saline solution or tap water.

In a series of experiments, the antisera to virus-sap and normal sap were drawn from the marginal ear-vein of the rabbit and allowed to stand on the clot overnight. The sera were tested on the following day in the active state, and after inactivation for one-half hour at 56°C., both with and without the addition of small amounts of fresh, normal, rabbit or guinea-pig serum (alexin).

The results of 6 separate experiments are summarized in Table IV.

Analysis of the Findings.—An examination of Table IV will show that:

Of 89 plants inoculated with virus-sap mixed *in vitro* with antiserum to virus-sap, 75 remained healthy.

The 26 corresponding control plants inoculated with the same amount of virus-sap in saline solution or tap water developed 100 per cent mosaic disease.

Of 20 plants inoculated with virus-sap mixed *in vitro* with antiserum to normal tobacco sap, only 6 remained healthy.

Twenty-seven of 29 plants inoculated with virus-sap mixed *in vitro* with normal rabbit or guinea-pig serum developed mosaic disease.

Discussion.—In respect to the power of inactivation of virus-sap exhibited by the antiserum to virus-sap, in comparison to that shown by antiserum to normal sap, and normal rabbit or guinea-pig serum, the results are so significant as to require no comment.

Several hundred plants were inoculated in these experiments, but since the concentration of virus varies with every preparation of virus-sap, the amount of antiserum required for complete inactivation cannot be determined in advance. The results of experiments in which a considerable excess of virus-sap was used are not reported in this paper. There is also some evidence to show that an excess of antiserum to virus-sap may inhibit an inactivation of the virus.

The results would seem to indicate further that antiserum to virus-sap, when heated for one-half hour at 56°C. (water-bath) loses some of its power to inactivate virus-sap (Table IV). The original strength of the antiserum can apparently be fully restored by the addition of rabbit or guinea-pig alexin. This behavior of the antiserum to virus-sap suggests that a lytic antibody for virus may be present in the antiserum. The importance of such a finding necessitates further efforts to corroborate these results.

Preliminary experiments indicate that the antiserum to virus-sap

of tobacco is also capable of inactivating virus-sap of tomato, pepper, and petunia plants.

SUMMARY.

1. Antisera were produced, separately, in rabbits to normal sap from healthy Turkish tobacco plants and to virus-sap from tobacco plants, affected with mosaic disease.

2. The immunologic reactions of the antisera were studied by means of:

(a) Alexin-fixation tests.

(b) Precipitation experiments, including: Precipitin-absorption tests with the same tobacco virus multiplied in tobacco, tomato, pepper, and petunia plants.

(c) Experiments with the inactivation properties of both antisera and normal serum on virus-sap.

3. From the results obtained from the above experiments, the following conclusions were drawn:

(a) Normal-tobacco-sap and virus-tobacco-sap possess antigenic substances in common.

(b) Normal sap and virus-sap of tomato, pepper, and petunia plants contain antigenic substances in common with normal sap of tobacco.

(c) Virus-saps of tomato, pepper, and petunia plants, have antigenic substances in common with virus-sap of tobacco, that are either not present in the normal tobacco sap or present only in small amounts.

(d) The two antisera possess alexin-fixing antibodies and precipitins in common.

(e) All of the precipitins to normal tobacco sap may be removed from either antiserum by absorption with virus-sap of tobacco.

(f) Specific precipitins for virus-sap of tobacco, tomato, pepper, and petunia are present in the antiserum to tobacco virus-sap and cannot be removed by complete absorption with normal sap of tobacco.

(g) Antiserum to virus-sap of tobacco, when used in an appropriate amount, has the power of completely inactivating virus-sap.

A corresponding quantity of antiserum to normal tobacco sap, or normal rabbit or guinea-pig serum, does not exhibit the same preventive action on virus-sap.

(h) There is some evidence that a specific antibody to virus-sap, lytic in nature, is present in the homologous antiserum.

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THE RELATION OF CHROMATIN TO HEMOGLOBIN AND BILIRUBIN.

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PLATE 44.

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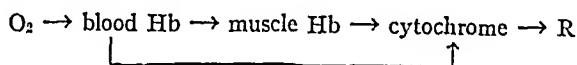
Recent studies of body pigment metabolism have given rise to two questions which await correlation. One is the origin of hemoglobin and the other that of the formation of bilirubin from other sources than hemoglobin. It is commonly accepted that blood pigment is the precursor of bilirubin, but Whipple (1) has suggested that hemoglobin and bilirubin may be formed independently of each other, probably in the liver, the former "by a progressive synthetic grouping of amino-acids, iron and other materials, which finally results in the finished product hemoglobin."

However there is evidence that hemoglobin appears in conjunction with the development of the erythrocyte and that it is derived from the chromatin of the hematopoietic cells. Schmidt (2), and more recently Sabin (3), have observed the presence of hemoglobin in the developing blood cells, although A. B. Macallum (4) first described its origin from the chromatin of the hematoblast in both amphibia and mammals.

Macallum devised methods of identifying cellular iron by micro-chemical methods and was able to distinguish the iron in hemoglobin from that in the chromatin of cells. His results showed that the first trace of hemoglobin in the earliest stages of an erythroblast was in the mitotic figure when the cell was dividing. These observations were based upon the fact that the iron in hemoglobin cannot be "unmasked" and specifically stained, while that of chromatin readily yields its iron in the presence of mineral acids.

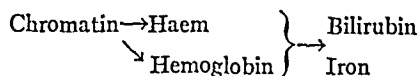
Since all the cells of the body contain chromatin, it is necessary to account for the fact that in these hemoglobin is not produced. Anson

and Mirsky (5) and Keilin (6, 7) have shown that all animal and vegetable cells (except anerobic bacteria) contain a respiratory pigment, called cytochrome, or haem. The function of this pigment is identical with that of hemoglobin and the following diagram illustrates Keilin's conception of the relation of cytochrome and hemoglobin to cell respiration in the body:



R being the oxidized substance in the cells.

The cytochrome or haem of the ordinary cell is then the analogue of the hemoglobin in the erythrocyte. It is logical to suppose that cytochrome is derived from the chromatin of the cells containing it and that on the death of the cell it yields bilirubin in the same manner as hemoglobin. A diagram illustrating this conception of the relation between the respiratory and bile pigments might be constructed as follows:



It is evident from this discussion that the theory of a primary "pigment complex" as expressed by Whipple (1) need not be postulated because chromatin itself wherever its location is probably the precursor of both hemoglobin and the respiratory pigment of the cells, and from these bilirubin is derived.

The relation of chromatin to hemoglobin formation could be strengthened further if it were possible to demonstrate a quantitative relationship between the rate of regeneration of hemoglobin to the chromatin content of the bone marrow cells in an animal with anemia due to hemorrhage. This possibility has been examined in work now to be described.

It was shown in a previous paper (8) that hemoglobin formation could be inhibited in an anemic animal by iron starvation. McMaster and Haessler (9) were able to show that body iron depletion causes a depression of red blood cell formation in the bone marrow. It follows then that by modifying the amount of iron available to the body cells one should be able to stimulate or inhibit bone marrow cell growth as well as hemoglobin formation.

Methods.

The general plan of study followed the methods previously described by Whipple and Robschey-Robbins (10), dogs being used as experimental animals. The bone marrow was studied first, during a period of rapid blood regeneration and second, during a period of relative iron starvation, while the peripheral hemoglobin level was held stationary. Blood regeneration was stimulated by feeding iron citrate or raw liver in relatively large amounts.

In the study of the relation of iron to hemoglobin formation in the bone marrow the methods described by Macallum were followed in making microchemical stains of the bone marrow cells. The marrow from the femur was chosen as representative because the decalcification of rib made the specimen unfit for the staining of iron. In each instance marrow from several parts of the bone were prepared and sections were cut also from the spleen and liver. The fresh tissue is fixed in 95 per cent alcohol, sectioned, and then placed in a solution of mineral acid and 95 per cent alcohol. Nitric, hydrochloric or sulphuric acid may be used, preferably the latter in 4 per cent concentration. After a short period (12 to 24 hours depending on the nature of the tissue) the sections are stained by applying a mixture of freshly made ammonium sulphide, and glycerine to the tissue and heating this preparation at a temperature of 55° for several days. The iron containing parts of the cell will then appear green in color while any substance containing iron not capable of being unmasked in the process will remain unstained. Of the several methods of demonstrating iron in tissue, it appeared after considerable experimentation that the ammonium sulphide-glycerine method was the most suitable. The sections were also stained with an aqueous solution of 0.5 per cent hematoxylin but the clearing of the section permitted by this method did not compensate for the lack of uniformity which they presented owing presumably to an inconstant hydrogen ion concentration (11). The steps in the preparation of tissue for examination were as follows:

1. Fixation in 95 per cent alcohol 24 hours.
2. Absolute alcohol 12 hours.
3. Xylol and paraffin.
4. Sectioning (6 M thick).
5. Place on slide without adhesive.
6. After drying dissolve paraffin in xylol and place in acid alcohol 24 hours with sealed cover.
7. Wash in 95 per cent alcohol thoroughly.
8. Place one drop of ammonium sulphide and one drop of glycerine on preparation, mix with glass rod and seal with cover slip.
9. Place preparation in warming oven 4 days.

Seven dogs were used in the experimental work but only the protocols are given of those from which photographs were taken. They were fed a bread mixture consisting of starch, bran, tomatoes, wheat flour, sugar and yeast. The iron content of the bread mixture was found to be 0.0055 per cent. The dogs were allowed only distilled water, and during the last 7 to 10 days of a period in which it was desired further to restrict the iron intake only milk was given the animals. These steps insured a rather high degree of iron restriction.

All the dogs had been continuously used in other experiments for a year prior

to sacrificing, and the hemoglobin had been maintained at a low level during this period. Dog 4 was fed 300 gm. of raw beef liver daily until hemoglobin production had reached a maximum. The dog was killed and the bone marrow removed by splitting the shaft of the femur and removing the bone marrow intact for hardening. In Dog 10 the hemoglobin level was receding without the necessity for hemorrhage at the time the animal was sacrificed, while in Dog 13 ferric citrate was used to increase hemoglobin production before studying the iron content of the bone marrow.

TABLE I.

Rate of Regeneration of Hemoglobin When Bone Marrow Was Studied.

Date	Red blood cell count	Oxygen capacity	Hb	Blood drawn	Hb	Hema- tocrit, per cent cells	Wt.	Diet
Dog 4								
1928	millions	cc.	per cent	cc.	gm.		kg.	Stock bread plus raw beef liver 300 gm. daily
Feb. 20		10.4	56.3	20	1.5	29	12.9	
" 23	6.54	11.1	60	20	1.5	30		
Mar. 1		10.6	57.3	100	7.9	30		
" 5		11.7	63.3	100	8.7	32	13.0	
" 7			Dog killed					
Dog 10								
Mar. 8		10.5	56.8	100	7.8	28	13.0	Whole milk only
" 15	5.52	9.5	51.4	15	—	24		
" 22		8.7	47.0	15	—	23		
" 22			Dog killed					
Dog 13								
Jan. 4	6.09	9.2	50	20		26		Bread plus Fe cit. 50 cc., 0.5 per cent
" 25		12.4	66	215	19.9	34		
Feb. 6			Dog killed					

The tissue blocks were cut at intervals during progress of the experiment, but staining procedures were carried out simultaneously on tissues on which comparisons of the staining reactions were to be made. The sections, usually four from each animal, were carried through the procedure to the warming oven at the same time, and when photographs were desired these were all made on the same morning for the entire set under identical conditions as to light, exposure, magnification, filters and development. Macallum found that the use of a steel blade in cutting

sections did not alter the staining depth although the handling of sections was carried out by means of glass rods always in glass containers. Study of individual cells was not undertaken since it was desired only to demonstrate the possible relation of rapid hemoglobin production to the amount of iron in bone marrow cells generally. It was necessary to observe in the sections that the green color due to iron was confined to the nuclei because any general diffusion of the color which occurred was evidence that the section was improperly prepared and must be discarded.

A direct estimation of the iron balance in dogs is impossible because of the large content of hair in the stools. However work in progress shows that a considerable depletion of the normal level of blood serum iron takes place under these conditions.

RESULTS.

The accompanying photomicrographs show the relative condition of the bone marrow with regard to its chromatin content. The sections demonstrated clearly that the iron content of the hematoblast is dependent on its chromatin content and that this in turn can be modified by iron feeding under the conditions of the experiments. In the animals in which the hemoglobin level is being barely maintained, nuclear chromatin stain is pale, while in those in which regeneration of hemoglobin was proceeding rapidly a heavy iron stain in the bone marrow is demonstrated.

The same phenomenon was present in sections from the spleen but in the liver the differences were not so manifest. As compared with the normal animal, however, all the tissues were poor in iron when anemia was present, and regardless of iron intake. Stains made of the liver and spleen of anemic dogs gave a much weaker chromatin reaction than those obtained from normal animals.

The interpretation of the depth of staining which different body cells exhibit after being subjected to the technique has been carefully considered. Since the chromatin is confined to the nuclei of the cells, the richness of its network is localized and the staining method itself prevents diffusion of iron into the surrounding tissues because water is not used in the preparation of the specimen. In order to further safeguard against mistakes in the subjective interpretation of the depth of staining only the two extremes in iron content were used. The bone marrow was either causing rapid production of hemoglobin or the hemoglobin level had remained stationary for a long period of

time under a constant stimulus. Hueck (12) has demonstrated a true comparison between the microchemical stain and chemical analysis of the tissues and his results have been confirmed in this laboratory by having a disinterested observer compare stained sections with the chemical analysis of tissues. The photomicrographs were made with the use of a red filter over a field large enough to include many cells.

The hypothesis that body cell chromatin, through cell metabolism, becomes the primary pigment complex is in no way antagonistic to the prevalent theories of pigment formation. It simply utilizes the facts already available and reduces the question to simpler and more basic considerations, whereupon investigation may continue.

SUMMARY.

1. Attention is directed to the diversity of opinion among investigators regarding the site and manner of hemoglobin formation in the body and its relation to bile pigment metabolism.

2. It is probable that in forming new hypotheses on this subject the earlier work of A. B. Macallum on the relation of chromatin to hemoglobin formation has not received sufficient consideration.

3. It has been shown by means of microchemical iron stains of the bone marrow cells, that the iron content of the hematoblast is increased during rapid hemoglobin production in simple anemia.

4. This fact is compatible with the work of Macallum who believed that hemoglobin is derived from the chromatin of the hematoblast. It does not support a theory that hemoglobin is formed as a part of a circulating pigment.

5. It is suggested that bilirubin is derived from the chromatin of body cells through the intermediary stages of the respiratory pigments, hemoglobin and cytochrome, from erythrocytes and other cells, respectively.

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EXPLANATION OF PLATE 44.

FIG. 1. Dog 13, fed Fe citrate. Bone marrow, femur showing increased density of cells. Preparations cannot be cleared for photographs. Red filter used. Oil immersion with No. 10 ocular. Sulphuric acid alcohol 24 hours. Ammonium sulphide-glycerine.

FIG. 2. Dog 10, iron starved. Same as above showing lessened density of bone marrow cells.

FIG. 3. Dog 4, fed liver. Showing moderate increase in chromatin network.



(Ricker: Relation of chromatin to hemoglobin and bilirubin)

EXPERIMENTAL DEHYDRATION: CHEMICAL CHANGES IN THE BLOOD OF THE DOG CONTRASTED WITH THOSE FOLLOWING OBSTRUCTION OF THE CARDIAC END OF THE STOMACH.

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Numerous factors are operative in the development of the toxemia characteristic of both simple and strangulation obstruction of the upper intestinal tract. Hartwell and Hogue (1) were the first to emphasize the importance of dehydration and to show that life can be prolonged by supplying water. We now consider in every case of intestinal obstruction dehydration as well as other factors, such as hypochloremia. It is difficult to decide just what part anhydremia plays in the toxemia. One also does not know how far other factors may be dependent on the dehydration.

Experimental dehydration in the dog has been thoroughly studied over short periods of time by Keith (2). He found no marked changes in the urea nitrogen and carbon dioxide combining power of the plasma. The serum chlorides showed a constant increase averaging about 10 per cent. With the polyuria there was an increased urea and chloride output. On restoring the water balance sodium chloride was retained. Keith dehydrated his animals by injecting intravenously a 50 per cent solution of glucose or sucrose at the rate of 8 gm. per kilo of body weight over a period of 2 hours. Mackay and Mackay (3) made similar studies over a longer period on rabbits dehydrated with a hypertonic solution of sucrose. Blood chemical determinations on such animals from day to day showed a marked increase in blood urea. The rise in blood urea they ascribed largely to increased urea formation from accelerated tissue catabolism.

Dehydration as observed clinically in infants suffering from nutritional disturbances has been well studied by Uthman and others (4). The subject of anhydremia has been reviewed by Marriott (5). The characteristic features of dehydration are: an increase in viscosity, an increase in non-protein nitrogen due to both retention and accelerated protein destruction, decreased oxygen capacity, decreased blood volume and volume flow, impairment of renal function, a negative nitrogen balance, an increased destruction of body protein, and fever. Most of these

features are equally characteristic of upper gastro-intestinal tract obstruction. The increase in non-protein nitrogen was one of the first recognized chemical changes in the disease, and has been confirmed by all later workers (6). There is an increased nitrogen excretion (7) due to an increase in protein destruction. The viscosity is much increased (8) with necessarily an accompanying decrease in volume flow. The oxygen capacity is decreased (9) and renal function is impaired (10). Long ago Spiegler (11) and others demonstrated an increased nitrogen excretion after water deprivation due to accelerated protein breakdown.

The experiments herein recorded have been made to compare the changes observed in upper gastro-intestinal tract obstruction with those of simple dehydration and determine in how far the chemical changes in obstruction might be explained by dehydration alone. The dehydration was effected by making daily one or more injections of a 50 per cent sucrose solution, 8 cc. per kilo of body weight being given at each injection. Urine collections are most difficult to make in intestinal or pyloric obstruction. We have shown (12) that dogs with obstruction of the cardiac end of the stomach develop a typical toxemia even more marked than with intestinal obstruction. Such animals cannot vomit so urine collections can be satisfactorily made. For this reason the results in a series of such animals are compared with those of a group dehydrated with sucrose.

Method.

All operations were done under ether anesthesia with aseptic technique. The obstruction of the cardia was effected by ligating with tape. All injections were made slowly into the jugular vein. The non-protein nitrogen was determined by the method of Folin and Wu (13), the urea nitrogen by the Van Slyke and Cullen modification of the Marshall method (14), the CO₂ combining power by the method of Van Slyke and Cullen (15), and the chloride on the tungstic acid filtrate in the manner suggested by Gettler (16). The hematocrit reading was obtained by centrifuging 10 cc. of blood mixed with 2 cc. of 1.6 per cent sodium oxalate (17) at high speed for $\frac{1}{2}$ hour. The fibrin determinations were made on this oxalated plasma by the method of Foster and Whipple (18). The total protein of the serum was determined by the micro Kjeldahl method. The globulin was precipitated with 22.2 per cent sodium sulphate as suggested by Howe (19) and the albumen determined in the filtrate. The globulin was obtained by difference.

EXPERIMENTAL OBSERVATIONS.

The chemical findings in the blood and urine of five animals dehydrated with sucrose are shown in Table I. The animals survived three

to five days, the average duration of the experiment being 3.8 days. Four animals showed a terminal rise in non-protein and urea nitrogen. The CO_2 combining power showed little change. The blood chlorides were uniformly elevated, the average increase being 13.9 per cent. The fibrinogen content of the plasma was increased in all animals. The total protein of the serum showed an average increase of 18 per cent. This increase was largely in the albumen fraction. The plasma percentage as determined in the hematocrit tube showed a decrease in all animals although in three animals there was a terminal increase. The average output of urine was 1102 cc. for the duration of the experiment. The nitrogen output was variable. The chloride excretion was usually high.

The findings in five animals with obstruction of the cardiac end of the stomach are shown in Table II. All animals showed the characteristic rise in non-protein nitrogen and urea nitrogen. The CO_2 combining power showed little change. In three animals there was a slight fall in chlorides and in two a slight rise. The fibrinogen content of the plasma is uniformly markedly increased. The total protein of the serum shows in every animal a marked increase. This increase affects both the globulin and albumen fractions. The plasma percentage is decreased. All animals showed a diuresis with a rather high nitrogen and salt output.

DISCUSSION.

The comparative data in dehydration and in obstruction of the cardiac end of the stomach are summarized in Table III. Several significant facts are evident. The increase in non-protein nitrogen and urea nitrogen is much the same in the two groups, although slightly higher in obstruction with a shorter average duration of life. The chlorides show the characteristic rise with dehydration. There is a slight fall with obstruction. The fibrinogen figures also show a marked difference in the two groups. With dehydration the average final determination is approximately double that of the initial determination; with obstruction the fibrinogen value is approximately quadrupled in a shorter time. The average protein increase with dehydration is 18.6 per cent, with obstruction it is 33 per cent. The final

Chemical Findings in Blood and Urine of the Dog After Dehydration with Sucrose (8 cc. per kilo body weight of 50 per cent solution at each injection).

[illegible]

6-59	1	13.6	43.6	20.6	500	41.9	938	3.86	3.85	6.71	56	650	0.81	5.30	0.96	6.24	1 injection
	2		43.9	22.4	520	43.8	1112	2.66	4.09	6.75	56	308	0.30	0.92	0.70	2.16	1 "
	3	11.1	93.7	40.6	560	32.4	1571	3.43	4.38	7.81	48	150	0.58	0.87	1.92	2.88	1 "
	4	10.8	103.0	76.6	550	25.8	1822	3.45	4.88	8.34	56	0					Died soon after
6-60	1	11.2	33.7	10.27	490	29.6	943	2.18	3.40	5.63	55	774	0.74	5.70	0.96	7.73	1 injection
	2		27.8	14.9	510	32.4	872	2.03	3.61	5.65	54	86	0.34	0.29	2.80	2.41	1 "
	3	10.5	33.3	16.8	550	29.6	1028	2.35	4.21	6.56	54	0					Died soon after

TABLE II.
Chemical Findings in Blood and Urine of the Dog After Obstruction of the Cardiac End of the Stomach.

Dog No.	Day of experiment	Weight	Blood (amount per 100 cc.)										Urine				Remarks
			Non-protein nitrogen	Urea nitrogen	Chlorides	CO ₂ combining power	Fibrinogen	Globulin (serum)	Albumin (serum)	Total protein (serum)	Plasma volume	Amount	Chlorides	Nitrogen			
		kilo	mg.	mg.	mg.	vol. per cent	mg.	gm.	gm.	gm.	cc.	cc.	per cent	gm.	per cent	gm.	
6-61	1	16.0	30.3	12.1	520	32.4	651	3.9	4.0	7.20	54	110	0.90	0.99	0.41	0.45	Operation Died soon after
	2		71.0	43.9	480	31.5	1398	4.11	5.64	9.79	45	0					
6-62	1	10.0	31.6	19.6	440	28.7	600	2.71	4.80	7.51	42	870	0.51	4.44	0.41	3.57	Operation
	2		67.5	40.6	440	32.4	1112	2.33	5.71	8.03	35	92	1.01	0.93	1.32	1.21	
	3		163.0	82.2	450	27.7	2031	2.38	6.32	8.66	35	0					
6-63	1	10.1	28.9	14.0	430	40.0	571	1.78	4.73	6.51	48	260	0.57	1.48	3.78	9.83	Operation
	2		37.5	13.1	500	44.7	958	2.15	5.01	7.18	37	190	0.62	1.18	1.65	3.14	
	3		42.0	12.1	440	34.3	1993	1.87	5.46	7.35	34	260	0.34	0.88	0.25	0.65	
	4		114.0	71.9	410		2739	2.63	5.75	8.39	33	0					
6-65	1	9.5	31.9	11.2	450	34.3	923	3.55	3.56	6.91	58	480	0.89	4.27	0.62	2.98	Operation
	2		37.7	19.6	450	40.0	1606	4.23	4.20	8.43	46	200	0.69	1.38	0.38	0.76	
	3		70.5	39.7	470	34.3	2270	4.45	5.02	9.46	45	300	0.39	1.17	0.78	2.28	
6-69	1	9.0	26.1	12.6	470	36.2	1158			6.28	50	130	0.66	0.86	2.32	2.99	Operation
	2		26.9	7.5	460	36.2	2068			7.47	42	170	0.51	0.87	1.65	2.89	
	3		63.4	27.8	460	39.0	3465			9.43	50	0					

TABLE III.
Comparative Findings in the Blood and Urine of the Dog After Dehydration with Sucrose and in Obstruction of the Cardiac End of Stomach.

Experiment	Average weight	Number of experiment	Average duration of experiment	Blood (amount per 100 cc.)												Urine (average)			
				Non-protein nitrogen		Urea nitrogen		Chlorides (as NaCl)		CO ₂ combining power		Fibrinogen (plasma)		Total protein (serum)		Plasma volume		Total amount	Per kilo
				Initial	Final	Initial	Final	Initial	Final	Initial	Final	Initial	Final	Initial	Final	Initial	Final		
Dehydration with sucrose.	10.5	5	3.8	36.1	80.7	11.7	50.4	478	511	32.8	29.2	922	1809	6.13	7.27	56	57	1102	105
Obstruction of cardiac end of stomach.....	10.9	5	3.0	29.8	96.4	13.9	53.1	462	451	31.2	32.8	581	2381	6.88	9.15	50	42	621	57

plasma volume is less with obstruction than with dehydration. The average total urine output for the duration of the experiment is 1102 cc. or 105 cc. per kilo with dehydration as contrasted with 621 cc. or 57 cc. per kilo in obstruction. At autopsy the stomach and intestinal tract of the animals with obstruction of the cardia contained only a minimal amount of fluid.

These results show that with a much less marked anhydremia the animals with obstruction of the cardiac end of the stomach present a much more marked chemical evidence of toxemia than animals simply dehydrated with sucrose. These facts indicate that there must be some factor besides dehydration in the characteristic toxemia of cardiac obstruction. This generalization must apply also to pyloric and high intestinal obstruction.

SUMMARY AND CONCLUSIONS.

A comparative chemical study of the blood and the urine of the dog with experimental dehydration and with obstruction of the cardiac end of the stomach is reported.

The average duration of life is slightly longer with dehydration than with obstruction.

The urine output per kilo of body weight is almost twice as great in dehydration as with obstruction.

The increase in non-protein nitrogen and urea nitrogen is much the same in the two groups although somewhat more marked with obstruction.

The chlorides of the blood are markedly increased with dehydration and slightly decreased with obstruction.

The increase in fibrinogen and total protein is twice as great with obstruction as with dehydration.

These findings indicate that there must be some factor or factors in addition to dehydration producing the toxemia of cardiac obstruction.

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CHEMICAL FINDINGS IN THE BLOOD OF THE DOG AFTER CLOSED-LOOP OBSTRUCTION OF THE JEJUNUM.

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The chemical changes in the blood of man and the experimental animal after simple intestinal obstruction are well established. There is typically a rapid fall in chlorides, usually a coincident rise in the CO_2 combining power, and a later increase in the non-protein nitrogen and urea nitrogen (1). In such an obstruction there is probably no absorption of toxic bodies, the product either of bacterial action or of digestive activity, from the lumen of the intestine. However, there may be a disordered function of the mucosa.

In strangulation obstruction other possible factors in the characteristic toxemia are added to those encountered in simple obstruction. As a result of the interference with the blood supply of the obstructed loop toxic bodies from the autolysis of the strangulated tissue as well as from bacterial and digestive activity may be absorbed. Wangenstein and Waldron (2) have shown that the products of the autolysis of the small intestine are very toxic. Mann (3), and Mason and Davidson (4) had previously demonstrated a marked difference in the toxicity of the products of autolysis of different organs when allowed to autolyze within the abdominal cavity. Liver is very toxic while the spleen is relatively little so. Whipple (5) has shown also the very marked toxic effect of the autolyzing mucosa of the small intestine after exposure to the x-ray.

The experiments recorded here have been made to compare the chemical findings in the blood of the dog with closed-loop obstruction of the upper jejunum with those characteristic of simple obstruction at the same level. In these experiments there is no interference with the blood supply. With such closed loops there is the best of opportunity for the absorption of the products of digestive and bacterial activity due to the pressure which always develops as the loop dis-

TABLE I.

Chemical Findings in Blood of Dog with Closed-Loop Obstruction of Jejunum.

Dog No.	Day after operation	Blood			CO ₂ combining power
		Amount per 100 cc.			
		Non-protein nitrogen	Urea nitrogen	Chlorides (as NaCl)	
		mg.	mg.	mg.	vol. per cent
4-93	0	28.2	14.0	490	39.0
	1	118.0	58.8	380	50.2
5-04	0	20.2	9.8	480	36.2
	1	20.3	10.5	410	41.9
	2	20.3	10.5	450	34.3
	3	34.9	17.7	350	38.1
	4	74.3	37.8	270	—
5-18	0	32.6	17.5	520	36.2
	1	40.0	25.2	460	43.8
	2	41.9	23.1	450	42.8
	3	238.0	127.2	440	40.0
	4	234.0	125.3	320	—
5-17	0	36.6	19.6	410	53.9
	1	37.5	19.6	320	44.7
	2	77.3	36.4	330	25.6
	3	152.0	82.0	320	28.7
4-96	0	22.4	11.2	470	38.1
	1 a.m.	36.9	19.6	390	48.3
	p.m.	36.9	18.6	350	45.7
	2 a.m.	45.7	25.9	320	52.0
	p.m.	78.0	40.6	300	60.5
	3 a.m.	84.3	42.0	290	65.3
	p.m.	205.0	102.2	270	65.3
	4 a.m.	246.0	121.2	220	67.1
	p.m.	246.0	122.6	220	68.1
4-95	0	20.2	4.2	460	38.1
	1 a.m.	43.6	21.7	400	43.8
	p.m.	48.9	24.5	410	44.7
	2 a.m.	45.3	23.1	370	50.2
	p.m.	58.0	29.4	330	50.2
	3 a.m.	103.0	75.7	250	38.1

tends. Almost all animals also show quite marked necrosis of the mucosa. This type of obstruction has, of course, no clinical analog.

Method.

All operations were done under ether anesthesia with aseptic technique. A loop of upper jejunum 8 to 10 inches in length was isolated and the ends closed by inverting and suturing the closed ends. The continuity of the intestine was restoring by making a lateral anastomosis. The animals were allowed water *ad libitum* but were given no food during the course of the experiment. Blood was obtained from the jugular vein for chemical analysis before operation and at 12 or 24-hour intervals until the end of the experiment. The non-protein nitrogen was determined by the method of Folin and Wu (6), the urea nitrogen by the Van Slyke and Cullen modification of the Marshall method (7), the CO₂ combining power by the Van Slyke and Cullen method (8), and the chlorides on the tungstic acid filtrate in the manner suggested by Gettler (9).

EXPERIMENTAL OBSERVATIONS.

The blood chemical findings in 6 dogs with closed-loop obstruction are shown in Table I. All animals showed the changes typical of simple high intestinal obstruction. The fall in chlorides is well marked in all. The increase in non-protein nitrogen and urea nitrogen which takes place in intestinal obstruction in a very short time is well shown in this series of experiments. The non-protein nitrogen of the blood of Dog 4-93 rose from 28.2 mg. per 100 cc. to 118 mg. within 24 hours after the obstruction was made. In Dog 4-96 the non-protein nitrogen increased from 84.3 mg. to 205 mg. per 100 cc. between the morning and afternoon determinations. Such increases can be explained only on the basis of a great acceleration of protein destruction.

At autopsy the closed loop was always markedly dilated, and usually discolored. In some there was marked destruction and desquamation of the mucosa of the loop. No animals are included in which there was peritonitis or evidence of leakage from the loop. Usually there was a great deal of vomiting from the onset of the experiment. In each animal the lateral anastomosis was functioning so the continuity of the lumen was assured.

DISCUSSION.

These results only emphasize that the changes characteristic of simple intestinal obstruction also take place when a closed intestinal

loop is made with a lateral anastomosis for resumption of continuity. The increase in non-protein nitrogen is rapid and most marked. The animals live a shorter time than with simple obstruction. The increased absorption and possibly disordered function of the mucosa which must take place, only accelerate the toxemia and the characteristic chemical changes.

SUMMARY AND CONCLUSIONS.

The chemical findings in the blood of 6 dogs with closed-loop obstruction of the upper jejunum are reported.

The duration of life with closed loops is less than with simple obstruction.

All animals showed a marked rise in non-protein nitrogen and urea nitrogen, and fall in chlorides. Usually the CO_2 combining power of the plasma is increased.

The findings in closed-loop obstruction are essentially the same as in simple intestinal obstruction.

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INTRADERMAL VERSUS SUBCUTANEOUS IMMUNIZATION OF MONKEYS AGAINST POLIOMYELITIS.

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Early experiments of Flexner and Lewis (1), Levaditi and Landsteiner (2), and Römer and Joseph (3) showed that monkeys once recovered from poliomyelitis are immune to subsequent intracerebral inoculations of poliomyelitis virus. This immunity was apparent no matter how slight had been the symptoms of the initial infection. The refractory state was of long duration and was absolute within the limit of infecting doses employed. These same workers noted that sera of convalescent monkeys, when mixed with poliomyelitis virus *in vitro*, rendered the material, otherwise infective, inactive when introduced intracerebrally in test animals.

These observations became the basis for numerous efforts to immunize animals against experimental poliomyelitis. Flexner and Lewis (4) injected monkeys subcutaneously with living active virus, beginning with a dose of 0.05 cc. This amount was given daily for four days and the series was repeated twice with a four day rest period between each individual set of injections. After the last interval the animals received on successive days 0.1, 0.5, and 1.0 cc. of virus, and after one month 5.0 cc. A week's rest period then followed, after which time the animals were tested intracerebrally with 2.0 cc. of fresh Berkefeld filtrate of poliomyelitis virus. Control monkeys which received 0.1 to 0.01 cc. of a similar filtrate intracerebrally developed typical poliomyelitis, whereas the vaccinated animals remained free from symptoms.

Levaditi and Landsteiner (5) attempted to immunize monkeys by a single subcutaneous inoculation of 0.5 cc. of virus suspension previously heated to 50°C. for 30 minutes. They failed to produce any immunity by this treatment. In another experiment glycerinated virus was heated to 50°C. for 2 hours; this heated virus was still active in producing disease when inoculated intracerebrally, but did not infect when given daily in subcutaneous doses of 2 cc. each over a period of one month. Nine days after the last subcutaneous injection two treated monkeys were tested intracerebrally. One of these showed slight prodromal symptoms of

poliomyelitis and the second gave no evidence of disease, whereas the control developed typical poliomyelitis.

Kraus (6) attempted the attenuation of poliomyelitis virus by phenolization and found that virus treated by 1 per cent phenol was rendered ineffective in four days, even when inoculated subdurally. He then endeavored to immunize monkeys by subcutaneous injection of 5 to 10 cc. of virus treated with varying concentrations of phenol for different periods of time. Of fifteen animals subjected to intracerebral test inoculations, twelve were immune. It is interesting to note that three animals immunized with virus treated with 1.5 per cent phenol for five days—a procedure calculated by Kraus to render virus inactive—were completely protected. In a second communication Kraus (7) reports results on two animals, one of which received 5 cc. of fresh virus cord emulsion subcutaneously, followed fifteen days later by 6 cc. of 0.5 per cent phenolized cord; a second monkey received 6 cc. of 0.5 per cent phenolized cord. Ten days later both monkeys resisted a test intracerebral inoculation with paper filtrate, whereas a control developed the typical disease.

Olaf Thomsen (8) gave monkeys daily sub-infective inoculations subcutaneously for twelve days and subsequently at weekly intervals, 0.06, 0.2, 0.4, 1.0, and 2.0 cc. of virus suspension. All animals were then resistant to intracerebral test but the author states that every animal showed symptoms such as excitement, tremor, and ataxia, during immunization. A second group was treated, using considerably smaller immunizing doses; of this series no animal showed symptoms during the immunizing procedure, yet all resisted test inoculation. The initial immunizing dose in this second series was only one hundredth of the estimated intracerebral infecting dose.

Zappert, Wiesner, and Leiner (9) attempted to immunize four monkeys by means of subcutaneous injections of gradually increasing doses of active virus emulsions. During the immunization, two of the animals died of intercurrent infection, one of typical poliomyelitis, and one of a supposedly marantic type of the disease. They attempted to induce an artificial immunity in one animal by the use of phenolized virus. The monkey developed the disease during the treatment designed to immunize against it.

Flexner and Amoss (10) described a so-called immunizing strain of poliomyelitis virus. To free a contaminated glycerinated brain from organisms, the tissue was immersed in 0.5 per cent phenol for a few hours and replaced in glycerine. This procedure was repeated once. The animals were subsequently infected with 1.0 cc. of 10 per cent suspension, their disease running an average eleven day course. In a series of passages the virulence of the strain decreased until a point was reached where the monkeys showed very few symptoms,—such as ataxia, tremor, and slight convulsive seizures—recovered, and were subsequently immune to strong virus.

Abramson and Gerber (11) treated emulsions of brain and cord of poliomyelitic monkeys for four hours with 0.5 per cent formaldehyde; this material was infective

when introduced subcutaneously in monkeys. They then endeavored to immunize by heated virus. On five successive days monkeys were injected with cord emulsion; the emulsion was heated to 55°C. for thirty minutes the first and second day, to 45°C. for thirty minutes the third day, to 37°C. for thirty minutes the fourth, and was used without preliminary heating on the fifth day. The dose was 5 cc. on each day. Three weeks after treatment the animals were bled and their sera tested for its power to neutralize virus. Of eight sera, three neutralized, four led to prolonged incubation period, and one failed. Intracerebral tests indicated that five of the treated monkeys were resistant to three to six minimum lethal doses of virus, whereas three proved susceptible.

In another series of three monkeys, Abramson and Gerber gave daily injections subcutaneously of 5 cc. of 10 per cent cord emulsion previously heated to 55°C. for one hour. On intracerebral test three weeks later, all developed poliomyelitis; of the three sera tested, one monkey showed no symptoms and two a delayed incubation period but eventually developed the disease.

McKinley and Larson (12) inoculated monkeys intracerebrally with 0.15 cc. of filtrate of a mixture of 5 per cent emulsion of castor oil soap and virus emulsion. The animals remained well and later resisted intracerebral inoculation of 0.7 cc. virus filtrate. Four more monkeys received 4 cc. of the virus-soap mixture intraperitoneally; none developed poliomyelitis, whereas a control with virus alone became paralyzed in a typical manner. Eleven days after the intraperitoneal virus-soap treatment, all four monkeys were tested intracerebrally; three remained well and one developed poliomyelitis.

The largest and most varied series of tests of poliomyelitis immunization is that of Aycock and Kagan (13). These investigators attempted to immunize with virus attenuated by various methods. The old experiments of Kraus with phenolized virus were repeated using material treated with 1.0, 0.75, 0.50, and 0.25 per cent phenol. The mixtures were kept for seven days in the icebox. Monkeys were then given four injections every other day of from 8 to 10 cc., beginning with the 1.0 per cent phenolized virus, and ending with the 0.25 per cent. Of four animals so treated, two became paralyzed during the process of vaccination, one failed to resist intracerebral test inoculation, and one resisted. In a second experiment monkeys were injected subcutaneously with virus cords dried over caustic potash from one to twenty-six days. Two of six monkeys became paralyzed during treatment, two failed to show protection on intracerebral inoculation, and two proved resistant. Next, virus cord was exposed to different glycerol-water dilutions (5 to 50 per cent glycerol) for seven months at ice box temperature. Monkeys were injected daily subcutaneously, beginning with virus from 5 per cent glycerol and ending with 50 per cent glycerol. Three animals developed paralysis during immunization; three failed to resist an intracerebral test; one resisted. In another group, virus in agar was introduced subcutaneously in eight animals; the total virus emulsion given ranged from 20 to 96 cc. of 5 per cent suspension in

from three to seventeen injections; two animals became paralyzed during treatment, two failed to show subsequent immunity, and two resisted.

In a fifth experiment virus was introduced intracutaneously in from 1 to 2 cc. amounts but was distributed in 0.05 cc. blebs, thus making from twenty to forty piqures each day of inoculation. The total amount of virus injected ranged from 5 to 76 cc. in six to forty-three inoculations, given during a period ranging from fifteen days to five months. Twelve monkeys were used; one became paralyzed during treatment; one failed to resist intracerebral inoculation; ten resisted one intracerebral test, but of these, two failed to withstand a second such test. Serum from eight resistant monkeys neutralized virus twenty-one times; one monkey's serum protected in one test, although the animal itself was not immune to intracerebral test inoculation.

From the review of the literature, it is apparent that the results of experiments designed to immunize monkeys against poliomyelitis have been inconclusive. Two facts stand out clearly; first, that it is impossible to protect monkeys by the use of killed virus, and second, that a definite though inconstant resistance to poliomyelitis can be brought about by the intradermal and subcutaneous introduction of the living virus. It was therefore deemed advisable to compare the results of the two routes of inoculation in order to gain information as to their relative efficacy. The following experiments were carried out with this point in view.

Experimental.

Eight monkeys (Table I) were immunized by the intracutaneous route, following in general the procedure of Aycock and Kagan. The injections were made biweekly and the total amount of a single day's dosage (1.5 to 2.0 cc. of 5 per cent glycerolated virus) was distributed in some twenty small blebs. The duration of the immunizing period was variable, lasting from three to five months. The total amounts of virus administered ranged from 42 to 66 cc. Before intracerebral test inoculation, all animals were bled in order to test their sera for virus-neutralizing power. The test inoculations were made with fresh virus injected intracerebrally in doses of 0.5 cc. of 5 per cent suspension. During the immunization period all animals were observed daily in order to detect possible abortive symptoms of disease and were exercised to bring out masked weaknesses.

Eight more monkeys (Table II) were treated in an analogous fashion but received their immunizing virus subcutaneously instead of intracutaneously. The amounts of virus used and the time intervals were comparable with those of the intracutaneous series, and bleedings and test inoculations were done in the same manner. Both tests for active immunity and for passive serum protection were rigorously controlled. The results in the two series are best seen in the tables.

DISCUSSION.

The primary purpose of this series of experiments was to determine whether the intradermal or the subcutaneous introduction of poliomyelitis virus was most effective in protecting monkeys against virus inoculation. Reference to Tables I and II shows that the degree of immunity produced is strikingly in favor of the intradermal method. Of the eight animals subjected to that procedure, all but one showed slight symptoms of the disease when tested by intracerebral inoculation of an amount of virus sufficient to cause characteristic poliomyelitis in the controls. No animal, however, developed more than the mildest abortive symptoms, such as tremor or excitement. No definite paralysis developed in any instance, and no subsequent muscle atrophy was observed. These results are sharply at variance with those of intracerebral inoculation of the group of monkeys treated by subcutaneous inoculation of virus. Four of the eight animals of this series developed typical poliomyelitis which progressed to prostration in two instances, and to well-marked paralysis in the other two. The remaining four animals proved to be completely refractory to the intracerebral tests.

During the process of immunization, the animals were closely observed to determine whether or not they developed an abortive form of poliomyelitis which might explain the subsequent immunity to the disease. Wickman (14), during the Swedish epidemic of 1905, noticed a considerable number of cases in man, which showed slight, transient symptoms, without developing the outspoken disease. Caverly (15), in the Vermont epidemic of 1904, saw six children with fever, nausea, and convulsions, whose illness never progressed further. Medin (16) also observed such abortive cases. Aycock (17) mentions the possibility that mild attacks of poliomyelitis are responsible for the development of immunity. In view of these observations we were on the alert to detect slight symptoms referable to the treatment. However, no deviation from the normal was discovered. Subcutaneous inoculation of virus has in our experiments on eight animals failed to produce the disease, although it has given rise to poliomyelitis in the hands of others (Flexner and Lewis, Aycock, and Olaf Thomsen). As evidence of the relative safety of intradermal inoculation of virus, in experiments to be reported, as much as 16 cc. of virus suspension

TABLE I.
Intradermal Immunization.

Monkey	Immunization		Total virus inoculated, 5 per cent suspension	Strain	Intracerebral test			Result of test		Serum neutralization	
	Begun	Ended			Date	Amount	Strain	Tested animal	Control	Test	Control
Died of tuberculosis during immunization											
1	9/21/27	12/27/27	cc. 42	M.A.	1/19/28	0.5 cc. 5 per cent suspension	M.A.	No symptoms	Typical poliomyelitis Prostrate in 6 days	Pooled neutralized	Typical poliomyelitis Prostrate in 11 days
2	9/21/27	1/19/28	66	M.A.	1/19/28	0.5 cc. 5 per cent suspension	M.A.	Slight excitement			
3	9/21/27	1/ 3/28	48	M.A.							
4	3/ 5/28	5/21/28	42	M.A.	5/31/28	0.5 cc. 5 per cent suspension	Aycock	Slightly slow	Typical poliomyelitis Prostrate in 6 days	Neutralized	Typical polio
5	3/ 5/28	5/21/28	42	M.A.	5/31/28	0.5 cc. 5 per cent suspension	Aycock	Tremor and ataxia; weak deltoid		Neutralized	Typical polio
6	3/ 5/28	5/21/28	42	M.A.	5/31/28	0.5 cc. 5 per cent suspension	Aycock	Slightly slow		Neutralized	Typical polio

7	6/ 8/28	11/ 1/28	56	Aycock	11/20/28	0.5 cc. 5 per cent suspension	M.A.	Slow and excited	Typical polio- myelitis Paralyzed in 8 days	Neutral- ized	Typical polio
	6/ 8/28	11/ 1/28	56	Aycock	11/20/28	0.5 cc. 5 per cent suspension	M.A.	Excitement and tremor		Neutral- ized	Typical polio

TABLE II.
Subcutaneous Immunization.

Monkey	Immunization		Total virus inoculated, 5 per cent suspension	Strain	Intercerebral test			Result of test		Serum neutralization	
	Begun	Ended			Date	Amount	Strain	Test animal	Control	Test	Control
9	10/27/27	1/27/28	45.9	M.A.	2/11/28	0.5 cc. 5 per cent suspension	M.A.	No symptoms	Prostrate 9th day	Pooled neutralized	Paralyzed 17th day
10	10/21/27	1/27/28	42.8	M.A.	2/11/28	0.5 cc. 5 per cent suspension	M.A.	No symptoms			
11	10/27/27	1/27/28	38	M.A.	2/11/28	0.5 cc. 5 per cent suspension	M.A.	No symptoms			
12	3/ 5/28	5/21/28	42	M.A.	5/31/28	0.5 cc. 5 per cent suspension	Aycock	Prostrate 10th day	Prostrate 11 days	Neutralized	Typical polio
13	3/ 5/28	5/21/28	42	M.A.	5/31/28	0.5 cc. 5 per cent suspension	Aycock	Paralyzed 18th day. Recovered		Serum contaminated	
14	3/ 5/28	5/21/28	42	M.A.	5/31/28	0.5 cc. 5 per cent suspension	Aycock	Prostrate 9th day		Typical polio	

15	6/ 8/28	10/16/28	56	Aycock	11/20/28	0.5 cc. 5 per cent sus- pension	M.A.	No symp- toms	Pro- strate in 7 days	Not done		Typical polio
										Neutral- ized		
16	6/ 8/28	10/16/28	56	Aycock	11/20/28	0.5 cc. 5 per cent sus- pension	M.A.	Paralyzed on 9th day. Re- covered				

has been given intracutaneously at one time without producing symptoms, while 0.005 cc. of Berkefeld filtrate of virus of the same strain inoculated intracerebrally consistently produced characteristic poliomyelitis in six days.

The question of the degree of protection conferred by the treatment proved to be an extremely interesting one. It has often been observed that different strains of poliomyelitis virus vary markedly in their power to produce the disease in susceptible animals. We therefore attempted to detect degrees of immunity by testing animals by intracerebral inoculation, not only with virus of the strain with which they had been immunized, but also with other strains. Thus monkeys treated with the M.A. strain of virus were tested with a fairly recent virus isolated in Vermont by Aycock, and animals immunized with Aycock strain were tested with the M.A. virus. The difference in the results is well-marked; monkeys treated in exactly the same way proved totally resistant to the relatively weak M.A. virus and not totally immune to the stronger Aycock virus. A group of three animals immunized with M.A. strain is described in Table III. All withstood subsequent intracerebral inoculation with both M.A. and Aycock virus but one of the three developed typical poliomyelitis on inoculation with a very active pooled, mixed virus derived from material of the original M.A. and K. strains which had been preserved in glycerol since 1920 (18, 19, 20).

That the immunity induced in the monkeys in these experiments is relative only, is more strikingly shown by tests employed to determine the power of the sera to neutralize the virus. The results of these determinations are shown in Table IV. The sera of Monkeys 1, 2, and 3 were pooled in one and those of 9, 10, and 11 in a second group. These two mixed sera neutralized, as was to be expected, since on intracerebral test the monkeys had proved resistant. Moreover, Sera 6, 7, and 8, derived from monkeys which had presented definite symptoms of poliomyelitis on intracerebral inoculation, were found also to neutralize completely a small, though ample dose, approximately 50 M.L.D. of a highly active virus filtrate of the pooled mixed virus strain. The results of the neutralization tests of sera 12 and 16 are especially significant. Although the monkeys from which they had come had proved ordinarily susceptible to intracerebral

TABLE III.
Results on *Reinoculation*.

Monkey	Intracerebral test				Result	
	Method of immunization	Immunization ended	Date	Amount	Strain	Test animal
				First intracerebral inoculation	Aycock	Prostrate on 30th day
2	Intradermal M.A.	1/19/28	6/11/28	0.3 cc. 5 per cent suspension	Aycock	No symptoms
	Subcutaneous M.A.	1/27/28	6/11/28	0.3 cc. 5 per cent suspension	Aycock	No symptoms
9	Subcutaneous M.A.	1/27/28	6/11/28	0.3 cc. 5 per cent suspension	Aycock	No symptoms
10						
				Second intracerebral inoculation		Prostrate on 7th day
					Pooled mixed virus	No symptoms
2	Intradermal M.A.	1/19/28	12/ 5/28	0.2 cc. 5 per cent suspension	Pooled mixed virus	Prostrate on 12th day
	Subcutaneous M.A.	1/27/28	12/ 5/28	0.2 cc. 5 per cent suspension	Pooled mixed virus	No symptoms
9	Subcutaneous M.A.	1/27/28	12/ 5/28	0.2 cc. 5 per cent suspension	Pooled mixed virus	No symptoms
10	Subcutaneous M.A.	1/27/28	12/ 5/28	0.2 cc. 5 per cent suspension	Pooled mixed virus	No symptoms

TABLE IV.
Serum Neutralizations.

No.	Treatment		Test		Result	Neutralization			Result		
	Dose	Virus	Date	Virus		Virus treatment	Route	Amount serum	Test	Control	
Intradermal											
1	42	M.A.	Died intercurrent infection		0.3 M.A. Pooled	2 hrs. incubator. Over- night icebox	Icer.	cc. 0.9	No symp- toms	Typical polio	
2	66	M.A.	1/19/28	M.A.							No symptoms
3	48	M.A.	1/19/28	M.A.							Slight excite- ment
4	42	M.A.	5/31/28	Aycock	Slow						
5	42	M.A.	5/31/28	Aycock	Tremor, ataxia, weak deltoid						
6	42	M.A.	5/31/28	Aycock	Slow						
7	56	Aycock	11/20/28	M.A.	Slow and ex- cited						
8	56	Aycock	11/20/28	M.A.	Excitement and tremor						
					0.1 M.V.		Icer.	0.9	No symp- toms	Typical polio	
					0.1 M.V.		Icer.	0.9	No symp- toms	Typical polio	
					0.1 M.A.		Icer.	0.9	No symp- toms	Typical polio	
					0.3 M.A.		Icer.	0.9	No symp- toms	Typical polio	
					0.2 M.V.		Cist.	0.8	No symp- toms	Typical polio	

Subcutaneous

9	45	M.A.	2/11/28	M.A. 0.5	No symptoms	0.3 Pooled	2 hrs. incuba- tor, Over- night icebox	Iccr.	0.9	No symp- toms	Typical polio
10	42	M.A.	2/11/28	M.A. 0.5	No symptoms						
11	38	M.A.	2/11/28	M.A. 0.5	No symptoms			Iccr.	0.9	No symp- toms	
12	42	M.A.	5/31/28	Aycock 0.5	Prostrate	0.1 M.V.		Iccr.	0.9	Died intercurrent dis- ease. Serum con- taminated	
13	42	M.A.	5/31/28	Aycock 0.5	Paralyzed	0.1 M.V.					
14	42	M.A.	5/31/28	Aycock 0.5	Prostrate	0.2 M.V.		Cist.	0.8	Late polio	Typical polio
15	56	Aycock	11/20	M.A. 0.5	No symptoms	Not done					
16	56	Aycock	11/20	M.A. 0.5	Paralyzed	0.2 M.V.		Cist.	0.8	No symp- toms	Typical polio

inoculation of the active pooled virus, their sera neutralized the same potent material. It is interesting to note that in one instance of the 16 animals tested did the serum fail to exhibit neutralizing power.

The results of the serum neutralization tests show that degrees of immunity to poliomyelitis virus exist not only in monkeys but suggest that the same condition exists in man. The employment of relatively small doses of filtrate of a virus strain, whose potency is quite constant, brings out degrees of specific protection in monkeys. Such variations would have been totally obscured by the ordinary means of determining immunity by the intracerebral inoculation of considerable amounts of suspension of virus possessing varying degrees of infective power. It is conceivable that the past failures of certain efforts to induce immunity in monkeys may be explained, not by the inadequacy of the methods employed, but rather by the overwhelming inoculation which the animal was required to withstand, doubtless far greater than that to which any human would be exposed. The intracerebral test inoculation particularly, with its associated damage to nervous tissue, makes demands upon the immune reaction of an animal many times greater than that arising in any natural method of infection.

CONCLUSIONS.

1. The introduction of considerable amounts of living, active poliomyelitis virus into the skin and subcutaneous tissue of monkeys protects the animals against intracerebral inoculations of similar virus material.

2. The degree of protection conferred by intradermal is greater than by subcutaneous injection.

3. During intradermal and subcutaneous inoculations, no local or general pathological signs were observed.

4. The degree of protection produced by the immunization methods used is not absolute, since a percentage of the inoculated monkeys respond to intracerebral injections of highly potent virus.

5. The sera of the animals inoculated intradermally or subcutaneously neutralized poliomyelitis virus *in vitro*, irrespective of the result of intracerebral inoculation, in all except one instance.

6. The power of the serum of treated monkeys to neutralize virus

in vitro is a more delicate test of immunity than is the intracerebral inoculation.

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STUDIES ON SOUTH AMERICAN YELLOW FEVER.*

I. THE STRAINS OF VIRUS IN USE AT THE YELLOW FEVER LABORATORY IN BAHIA, BRAZIL.

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Up to the time of writing, the middle of January, 1929, there have been five strains of yellow fever virus studied in *Macacus rhesus* monkeys at the laboratory in Bahia. One of these strains came from Rio de Janeiro, three were picked up from local cases of yellow fever, and one was first established in Africa.

In order that references to these viruses in future publications may be more comprehensible, it is our purpose to state briefly how we came into possession of the strains.

F. W. Strain.—This strain was received from Rio de Janeiro through the kindness of Dr. Aragão. The human patient, F. W., was a Hungarian, male, 16 years old. He proved to have a mild attack of the disease. Blood was taken approximately 48 hours after onset. The virus had gone through four passages before being given to us.

Early in September, 1928, Dr. J. H. Bauer brought to Bahia blood and tissues from fifth and sixth passage monkeys which he had inoculated in Rio de Janeiro. He also brought mosquitoes which had fed on these monkeys. Among the animals inoculated in Bahia after his arrival, two showed temperature curves suspiciously like those of mild yellow fever. However, direct transfers from these animals were negative; mosquitoes fed on one were definitely proved non-infective and those fed on the second were doubtfully infective.

Previous to Dr. Bauer's return to Bahia, blood and tissues had been received by airplane. At the time it was thought that the results of inoculating this material were negative. One animal showed a temperature of 104.8° on the fourth day, but after lancing a superficial abscess at the point of inoculation the fever dropped. However, at the height of the fever a batch (No. 6) of *Aedes aegypti* was fed on

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TABLE I.
Fatal Infections of Monkeys after Inoculations with Brazilian Yellow Fever Virus.

Strain	<i>M. rhesus</i> No.	Temperatures, a.m. and p.m.										Remarks	
		Day of inocul.	1	2	3	4	5	6	7	8	9		10
B. B.	6	— 103.2	103.8 103.4	103.0 103.6	103.8 102.3	104.9 105.3	101.9 Dead						Previously used animal. Inoculated with 3 cc. liver emulsion from <i>rhesus</i> No. 180
"	8	103.4 103.7	103.6 102.2	102.6 103.8	104.4 101.9	Dead							Previously used animal. Inoculated with 4 cc. blood mixture from <i>rhesus</i> Nos. 171, 173, 180
"	12	— 102.9	103.1 103.2	102.0 102.5	102.9 104.7	103.8 104.0	Dead						Previously used animal. Inoculated with 3 cc. liver emulsion from <i>rhesus</i> No. 180
"	14	103.0 103.4	103.1 102.6	103.7 103.9	103.0 102.3	Dead							Previously used animal. Inoculated with 4 cc. blood mixture from <i>rhesus</i> Nos. 171, 173, 180. No fever, but autopsy lesions typical
"	23	103.4 103.6	102.9 103.0	103.2 103.2	102.6 102.6	Dead							Previously used animal. Inoculated with 4 cc. blood mixture from <i>rhesus</i> Nos. 171, 173, 180. No fever, but autopsy lesions typical
"	24	— 103.0	102.4 101.9	102.7 102.4	103.9 105.1	96.8 Dead							Previously used animal. Inoculated with 3 cc. blood-liver mixture from <i>rhesus</i> Nos. 86, 188, 192. Sacrificed when moribund
"	47	— 102.9	104.4 104.3	103.9 103.3	104.9 103.8	Dead							Previously used animal. Inoculated with 3 cc. blood-liver mixture from <i>rhesus</i> Nos. 86, 188, 192. Sacrificed when moribund
"	51	— 103.4	103.7 103.2	103.4 102.4	103.9 104.0	103.9 104.3	95.7 Dead						Previously used animal. Inoculated with 3 cc. blood-liver mixture from <i>rhesus</i> No. 141. Sacrificed when moribund
"	72	— 103.7	103.0 103.2	104.7 105.0	105.0 104.5	103.0 98.5							Previously used animal. Inoculated with 2.5 cc. citrated blood from <i>rhesus</i> No. 166. Sacrificed on evening of 4th day, when moribund

No.	Sex	Inoculation schedule and results										Remarks
		1	2	3	4	5	6	7	8	9	10	
"	86	102 9	103 1	105 0	103 6							Previously used animal. Inoculated with 2.5 cc. citrated blood from <i>rhesus</i> No. 166. Sacrificed on evening of 4th day, when moribund
"	111	103 6	103 1	104 4	104 1	91 2						New animal. Inoculated with 5 cc. citrated blood from <i>rhesus</i> No. 107. Sacrificed when moribund
"	121	104 9	103 5	103 7	103 4	103 6	105 2	104 6	104 9	95 0		New animal. Inoculated 3 times with a total of 20 cc. from 4 animals
"	126	103 7	103 1	104 4	104 6	103 1	102 6	102 2				New animal. Inoculated with 8 cc. citrated blood from 4 animals. Sacrificed when moribund
"	128	102 9	103 8	103 1	103 6	104 5	102 9	102 0				New animal. Inoculated with 9 cc. citrated blood mixture from <i>rhesus</i> Nos. 121 and 125
"	131	102 8	104 1	103 9	103 4	104 1	93 0					New animal. Inoculated with 7 cc. blood from <i>rhesus</i> No. 116 (1st passage from S. R.). Sacrificed when moribund
"	136	103 8	103 9	103 0	103 9	106 0						New animal. Inoculated with 4 cc. citrated blood from <i>rhesus</i> No. 128
"	141	102 9	103 7	102 8	103 5	105 0	Dead					New animal. Inoculated with 3 cc. liver emulsion from <i>rhesus</i> Nos. 136 and 137
"	155	103 9	103 7	103 9	103 5	105 3	Dead					New animal. Inoculated with 8 cc. blood from <i>rhesus</i> No. 141. Autopsy showed tuberculo- sis also
"	158	103 1	103 4	104 2	106 0	104 2	103 9	102 0	Dead			Previously used animal. Inoculated with 3 cc. blood-liver mixture from <i>rhesus</i> Nos. 24, 47, 107
"	170	102 0	102 8	102 9	103 4	102 8	102 3					New animal. Inoculated with 10 cc. liver emulsion from <i>rhesus</i> No. 161. Liver frozen 10 days. Animal sacrificed when moribund on 5th day

Note: Animals 111 to 155, inclusive, were infected earlier chronologically than animals 6 to 86; apparently, the virus was more active when used in the later experiments.

Animals infected through mosquito transmission will be considered in a separate paper.

TABLE I.—Continued.

Strain	<i>M. rhesus</i> No.	Temperatures, a.m. and p.m.										Remarks	
		Day of inocul.	1	2	3	4	5	6	7	8	9		10
B. B.	180	—	103.6	105.4	104.6								New animal. Inoculated with 2.5 cc. citrated blood from <i>rhesus</i> No. 166. Sacrificed. Not moribund, but would not have lived until following day
"	188	102.9	103.8	104.5	102.4								New animal. Inoculated with 4 cc. blood mixture from <i>rhesus</i> Nos. 171, 173, 180
"	192	—	102.8	102.6	104.6	105.4							New animal. Inoculated with 2 cc. blood-liver mixture from <i>rhesus</i> Nos. 170, 180

this monkey. These mosquitoes, together with batch No. 7, fed on a sixth passage animal in Rio de Janeiro, were allowed to feed on *rhesus* No. 52 on Sept. 17. The following day the temperature of this animal reached 106.1°F. and it passed 104°F. daily for five days. On the seventh day the monkey was killed. The liver showed some fat and a thin scattering of necrotic cells. It was evident that the animal was on the road to recovery when sacrificed.

From mosquitoes fed on *rhesus* No. 52 the strain was maintained. Separate feeding and separate injection of mosquito emulsion of batches Nos. 6 and 7 caused no rise of temperature in experimental animals. However, later tests indicated that *both* batches had caused immunity.

The F. W. strain in our hands has not proved very virulent. It sometimes gives rise to a rather severe temperature reaction, but it has caused death in only three instances.

B. B. Strain.—On Sept. 2, 1928, the writers were notified of a suspected case of yellow fever in the person of B. B., a Russian Jew, 16 years of age, who had been one year in Brazil. Blood was drawn at 10.30 a.m. and inoculated into two guinea-pigs and a *rhesus* monkey. The boy was said to have been taken sick at 10 a.m. on Aug. 30, although there was an indefinite history of indisposition since the evening of the 29th. At the time of our visit he lay hunched up in bed, rather dull, with pulse of 90 and temperature of about 103°F. The eyes were congested and slightly icteric. The tongue was pointed, had red margins, and was furred white on the dorsum. The lips were cracked and excoriated. He had complained of headache previously. There had been vomiting, but no black vomit. Urine was said to contain casts and 1 gm. of albumen per liter. Later he developed bleeding gums, and black vomitus appeared. It was clinically a frank case of yellow fever. On Sept. 4 he became semicomatose and died in the evening. No autopsy was obtainable.

On the seventh day after inoculation one guinea-pig developed a fever of 105.2°, but transfer to another animal was negative and blood cultures in semi-solid medium gave no growth. On the eleventh day the *rhesus* monkey (No. 31) had a temperature of 104.3° and transfer was made to another animal (No. 45). The latter showed fever on the seventh day. Third and fourth passage animals both had high fever on the third day and were sacrificed to obtain tissues for Dr. Bauer to take to New York. A fifth passage animal (No. 63) died, but with a complicating peritonitis. After the seventh passage direct transfers lost in virulence for a time, owing, probably, to shortage of animals and the necessity of using partially immune monkeys. The strain was established again in virulent form through mosquitoes. The batch fed on *rhesus* No. 63 caused a fatal infection when allowed to feed on *rhesus* No. 98. From this point the virus has been carried by a combination of direct transfers and mosquito transmission through a total of at least 14 passages. To the end of December, 1928, 135 animals had been inoculated with this strain, of which 26 had either died or been sacrificed when moribund. However, this small percentage of deaths does not give a true picture of the present virulence, since the total (135) includes all those animals used in building up the virulence, many

negative or partially successful mosquito transmissions, and a large number of animals inoculated with other material and later tested for immunity with the B. B. virus. Autopsies on fatal cases have shown the classical picture of yellow fever. (See reports by Hudson on African studies.¹)

S. R. Strain.—On Oct. 26, 1928, Dr. Eduardo de Araujo notified us that he had heard of a suspicious case of fever. However, the attending physician had not considered it sufficiently suspicious to report to the Health Department.

We found the patient, S. R., to be a Spanish woman, married, 18 years of age. She had become ill on the afternoon of Oct. 23, *i.e.*, approximately 66 hours before our visit. Fever had risen to 104°F., but was down to 100.8°, axillary. The eyes were not injected nor icteric. The tongue was slightly coated, but was not pointed, nor did it have the red margin frequently seen in yellow fever. There had been a little bilious vomiting, but no black vomit. The attending physician reported the urine to have albumen in considerable amount. The patient appeared to be in no pain, was bright and attentive, but not anxious. On the fifth day she was frankly convalescent, with normal pulse and temperature, without ever having shown jaundice or black vomit. The only suspicious signs had been the albumen in the urine and a rather high temperature.

Blood taken at the time of our visit (66 hours after onset) was inoculated into two guinea-pigs and two *rhesus* monkeys (Nos. 116 and 117). On the seventh day *rhesus* No. 116 showed a rise of temperature to 104.2°F. and blood transfer was made to *rhesus* No. 133. The latter had a fever on the day after inoculation but not again until the fourth day, when the temperature rose to 106.2°F. The animal died on the fifth day with typical gross and microscopic lesions of yellow fever. Mosquito batch No. 53 was allowed to feed on this monkey on both the first and fourth days. Blood transfer from the same monkey to *rhesus* No. 142 was made on the fourth day, and liver from the autopsy was emulsified and inoculated into *rhesus* No. 143. The two animals inoculated had no obvious reaction. On Dec. 1 only two stegomyias remained alive in batch No. 53. These engorged on *rhesus* No. 168 and the latter developed a fever of 104.4° on the fourth day, but recovered. Blood from this monkey (No. 168) was injected into *M. rhesus* No. 186, which died from other causes before showing any signs of yellow fever. The surviving two mosquitoes of batch No. 53, together with seventeen others whose remains were fished from the wet cotton and the pan of honey in the cage, were emulsified and inoculated into *rhesus* No. 177, without result. Mosquito batch No. 72 which had engorged on *rhesus* No. 168 was allowed to feed on *rhesus* No. 217. On the next day the temperature of this animal reached 104.0° and in the second day rose to 104.8°. Blood transfer to *rhesus* No. 228 proved positive.

The following animals which received S. R. virus have been proved immune to B. B. strain virus, having given no temperature reaction following the inoculation

¹ Hudson, N. Paul, *Am. J. Path.*, 1928, iv, 395.

of virulent material: No. 117 (one of the original blood inoculations), No. 143 (inoculated with emulsified liver), No. 168 (fed upon by mosquitoes of batch No. 53) and No. 177 (inoculated with emulsified mosquitoes of batch No. 53). *M. rhesus* No. 116, of the first passage of the S. R. strain, proved absolutely resistant to the African strain of virus.

J. V. O. Strain.—On Sept. 14, 1928, Dr. Barros Barreto came to tell us that a suspected case had been admitted to the Isolation Hospital. Later in the evening the patient, J. V. O., a young Spaniard, was seen by Dr. Bauer and the writers. Although only at the end of the third day of the disease, he was already comatose and had a normal to subnormal temperature. There was noted muscular twitching and bleeding from nose and rectum. It was considered useless to take blood for inoculation. He died at 5 a.m., Sept. 15, and the autopsy was started at about 9:30 a.m. by Dr. Eduardo de Araujo. Icterus was very marked in the dead body. Autopsy findings were typical of yellow fever, including sub-epicardial hemorrhages, bleeding into stomach and intestines, box-wood liver, and intensely injured kidneys.

Liver, spleen, and kidney tissues taken at autopsy were emulsified separately and three animals were inoculated. *Rhesus* No. 48 received spleen emulsion. A severe necrosis of the abdominal wall developed and the monkey was sacrificed on the fifth day. There was no evidence of yellow fever. *Rhesus* No. 47 received liver emulsion. A small abscess appeared and there was a slight fever for two days. Blood transfer to a normal animal gave no results, and No. 47 recovered. On Dec. 10 this monkey was inoculated with virulent B. B. strain material and died on the fourth day with typical yellow fever. No immunity had developed.

M. rhesus No. 46 received kidney emulsion. Here also an abscess resulted. For four days following injection there was a fever, which upon one occasion reached 105.6°. On the second day blood transfer was made to *rhesus* No. 22, but culture of this blood made by Dr. Bauer, yielded a gram negative organism thought to be *B. coli*. *M. rhesus* No. 22 reacted with a high fever, but no further transfers were made, because we thought that the infection was bacterial; the animal recovered. The temperature of *rhesus* No. 46 again reached or passed 104°F. on the afternoons of the twelfth, fourteenth, and sixteenth days after inoculation. Suddenly on the morning of the twentieth day the temperature went up to 105°F. and the monkey was sacrificed. Blood culture at this time yielded a pure growth of streptococci. Both blood and liver were transferred to *rhesus* No. 44. The latter animal had previously been used for experimentation but was considered to be non-immune. Sections of the liver of *rhesus* No. 46 showed the deposition of considerable fat, but apparently no necrosis.

M. rhesus No. 44 developed an abscess at the point of inoculation and the temperature passed 104°F. on the first and third days. On the fourth day there was a rise to 105.9° and mosquito batch No. 29 was allowed to feed. No blood transfer was made because it was thought that the fever arose from bacterial infection. *M. rhesus* No. 44 proceeded to recover, with only one more marked rise in temper-

ature on the tenth day. On Dec. 8, *rhesus* No. 44 was inoculated subcutaneously with 3 cc. of liver emulsion from *rhesus* No. 180, B. B. strain. Not the slightest temperature reaction resulted, although control *rhesus* No. 188 died with typical yellow fever on the fifth day.

On Nov. 1 mosquito batch No. 29, which had fed on *rhesus* No. 44, was allowed to feed on *rhesus* No. 132. Four days later the whole batch (58 remaining mosquitoes) was killed, ground up, and inoculated into the same animal. On Nov. 11 there appeared a fever of 104.6°F. and blood transfer was made to *rhesus* No. 154. The latter had a fever on the second and third days. Blood transfer was made to *rhesus* No. 158, and mosquito batch No. 61 was fed. *M. rhesus* No. 154 later developed a more or less continuous fever and it was sacrificed; early tuberculosis was present. *M. rhesus* No. 158 had a slight fever, beginning on the seventeenth day after inoculation and appearing on three days. However, this was apparently not yellow fever, because ten days after the last febrile access the animal was inoculated with B. B. strain virus and succumbed on the sixth day.

On Dec. 1 mosquito batch No. 61, which had fed on *rhesus* No. 154, was allowed to feed on *rhesus* No. 174, and this monkey had a fever of 104°F. on the second day. Eleven days after the infective feed, virulent B. B. strain material (the same as used for No. 158) was inoculated but gave no reaction. This J. V. O. strain, carried in mosquitoes, has been dropped, since it appeared to be avirulent and of no use for experimental purpose.

On Sept. 15, the day of the death of J. V. O., the writers and their assistants captured 59 female *Aedes ægypti* in the bedroom where the patient had slept during his illness until removed to the Isolation Hospital. Upon three occasions, the first time eight days after capture, these mosquitoes were allowed to feed upon *rhesus* No. 60. Five days after the first feeding and three days after the second, this monkey's temperature reached 103.9°F. There was then a drop until two days later when 104.4° was reached. Blood transferred at this point gave no reaction. The mosquitoes which fed on No. 60 were allowed to engorge on one normal monkey without result, but were destroyed through an error before further experimentation could be carried out. The original mosquitoes caught in the bedroom of J. V. O. were eventually ground up and inoculated into two monkeys. These showed no fever and were later proved susceptible to B. B. strain virus. Evidently the virus, if present, was not sufficient either to infect or to immunize two animals. However, *rhesus* No. 60, which had shown a temperature reaction following bites, appeared to be resistant upon further inoculation.

African Strain.—This is the Asibi strain, which has been in use for many months at the laboratory of the West African Yellow Fever Commission of the Rockefeller Foundation in Lagos, Nigeria. It was later established by Dr. Sawyer in New York, sent from there to Rio de Janeiro in November, 1928, established at the Oswaldo Cruz Institute, and sent from Rio to us through the kindness of Dr. Aragão. In our hands it has gone through five passages and has killed eleven monkeys.

TABLE II.
Fatal Infections of Monkeys after Inoculation with African Yellow Fever Virus.

Fatal Infections of Monkeys after Inoculation.												Remarks	
Temperatures, a m and p m.													
Strain	U. rhombi No.	Day of inocul.	1	2	3	4	5	6	7	8	9	10	
Asibi	175	—	104 7	103 9	103 1	106 5	104 9						Inoculated with monkey liver sent from Rio de Janeiro. Developed abscess
"	185	103 4	104 1	103 9	103 9	106 0	103 3	Dead					Inoculated with 2 cc. blood from No. 175 (taken on 4th day)
"	190	—	103 9	104 0	Dead								Inoculated with 2.5 cc. citrated blood from No. 185. Sacrificed when moribund on evening of 3rd day
"	118	102 7	102 2	104 6	100 4								Previously used animal probably partially immune. Inoculated with 2.5 cc. citrated blood from No. 185
"	201	—	102 0	103 9	103 4	104 0	104 2	101 7	Dead				Inoculated with 2 cc. blood-liver mixture from No. 190
"	216	103 8	103 6	105 1	104 4	Dead							Inoculated with 10 cc. blood-liver mixture from No. 201. Blood 19 days old, liver 17 days old (frozen most of time). Sacrificed when moribund
"	229	102 9	103 0	103 6	102 9	103 8	103 9	99 1					Inoculated with 2 cc. citrated blood from No. 209 (the latter infected by mosquitoes). Sacrificed when moribund
"	204	102 7	103 4	103 5	103 0	103 6	104 8						Previously used animal. Inoculated with 2 cc. citrated blood from No. 209
"	189	—	102 5	103 6	104 8	104 6	105 8	102 0					Previously used animal. Inoculated with 2 cc. citrated blood from No. 209

DISCUSSION AND CONCLUSION.

It would appear that inoculation of *rhesus* monkeys served a diagnostic purpose in one of our cases, that of S. R. The disease was so mild that in the absence of an epidemic no clinician would have made the diagnosis without this laboratory procedure.

It should be pointed out that following inoculation of liver emulsion, and sometimes even of blood, it is impossible to tell whether an early fever resulting is due to a protein reaction, to bacterial infection, or to true yellow fever. If plenty of animals are available the only safe plan is to take blood for subinoculation and for cultures, and to feed mosquitoes. Blood may be kept frozen for some time before inoculation if it is found to be necessary, or if it is desired to await the outcome of the disease in the animal bled.

A combined blood and liver transfer showed the presence of virus in *rhesus* No. 46 twenty days after the original inoculation with kidney from the autopsy in the case of J. V. O. The same animal had had an infected body wall and a bacteriemia.

On the basis of later experience we can see some of our mistakes. In the second S. R. passage (*rhesus* No. 133) blood transfer should have been made at the time of initial fever, the day following inoculation. In the case of *rhesus* No. 60, mosquito infection from J. V. O., transfer probably should have been made when the temperature reached 103.9°F. on the fifth day. Two days later, with a fever of 104.4°F., the blood appeared to be non-infective. A temperature of 104°F. in monkeys is usually a safe borderline between fever and no fever, but seemingly not always.

Our experience with the South American viruses so far indicates that it takes much care, patience, and an abundance of monkeys to build up and maintain a high degree of virulence.

For invaluable help in securing these yellow fever strains, and for much assistance and advice in establishing the laboratory we gratefully acknowledge our indebtedness to Dr. J. H. Bauer of the West African Yellow Fever Commission of the Rockefeller Foundation, to Dr. Henrique de Beaufreire Aragão, of the Oswaldo Cruz Institute, Rio de Janeiro, to Dr. Antonio Luiz C. A. de Barros Barreto, Director of the Department of Health, State of Bahia, and to Dr. Eduardo de Araujo, Director of the Oswaldo Cruz Institute, São Salvador, State of Bahia.

STUDIES ON SOUTH AMERICAN YELLOW FEVER.*

II. IMMUNITY OF RECOVERED MONKEYS TO AFRICAN VIRUS.

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(Received for publication, March 19, 1929.)

The purpose of the present paper is to offer further proof of the identity of Brazilian and African yellow fever viruses. In addition to the clinical and pathological evidence, the literature at the present writing contains two reports indicating immunological identity of the strains. In June, 1928, Dr. Aragão (1) received African virus, brought from Paris by Dr. Carlos Chagas. This was apparently of low virulence and caused only a mild febrile reaction. However, the two inoculated animals later proved immune to a Brazilian strain. Recently a valuable paper by Theiler and Sellards (2) has presented the results of protection experiments in which immune sera from human cases of the Parahyba, Brazil, outbreak of 1926 were tested against African virus. Some of the sera were not potent, but others gave absolute protection.

On Dec. 1, 1928, we received African virus, Asibi strain, from the Oswaldo Cruz Institute in Rio de Janeiro, through the kindness of Dr. Aragão. The strain had previously been established in New York by Dr. Sawyer and was sent by him to Rio de Janeiro, arriving there early in November. The establishment of this virus in Bahia has enabled us to test the immunity of a number of animals recovered from Brazilian yellow fever.

The results are presented in Table I. In addition to four non-immune controls, three of which promptly died, twenty-two supposedly immune animals were inoculated. Of the twenty-two one died of typical yellow fever, although death occurred three days later than

* The studies and observations on which this paper is based were conducted with the support and under the auspices of the International Health Division of the Rockefeller Foundation.

TABLE I.
Results of Inoculation of African Virus in Monkeys Recovered from Brazilian Yellow Fever.

Rhesus No.	Original Brazilian strain	Temperatures Recorded, a.m. and p.m.																					
		Day of inoculation	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	
Experiment I. Inoculated with 2.5 cc. citrated blood subcutaneously from rhesus No. 185, African strain, on Dec. 7																							
190	Control	—	102.7	102.9	103.6	Sacrificed when in extremis, evening of 3rd day. Yellow Fever																	
		102.7	102.2	104.6	100.4																		
116	S. R.	—	102.2	102.3	101.9	102.2	102.7	102.6	102.6	102.4	102.2	102.1	102.6	102.0	102.2	102.0	102.0	102.1	102.3	102.2	102.0	102.4	
		103.0	102.7	102.0	102.0	102.4	102.8	102.6	102.9	102.4	102.6	102.4	102.4	102.7	102.5	102.1	102.5	—	102.5	—	102.2	—	
118	B. B.	—	102.0	103.9	103.4	104.0	104.2	101.7	Yellow Fever														
		101.8	102.3	103.7	103.2	103.6	105.0	102.0	Dead														
122	F. W.	—	104.0	102.6	102.1	101.7	102.1	102.0	102.4	103.6	103.8	102.9	103.2	103.1	103.0	103.1	102.0	102.6	102.5	102.4	102.6	102.4	
		104.8	104.0	102.0	102.8	102.0	101.9	101.3	103.9	104.9	103.8	103.0	103.4	103.3	103.2	103.2	102.8	—	102.7	—	102.8	—	
125	B. B.	—	102.6	103.0	103.4	102.2	102.6	101.9	102.0	101.5	102.4	102.1	102.0	101.9	102.0	102.1	102.2	102.2	102.6	102.2	102.3	102.5	
		103.0	102.0	103.6	104.0	102.0	101.7	101.5	101.9	102.0	102.2	101.9	101.7	102.1	101.9	102.4	102.4	—	102.4	—	102.6	—	
31	B. B.	—	102.7	102.4	102.8	102.5	102.0	102.3	102.6	102.9	103.2	103.4	103.2	102.9	102.8	103.2	101.6	103.2	103.1	102.9	102.6	103.5	
		103.0	103.0	102.6	103.0	101.8	101.6	101.7	102.8	103.0	103.6	103.4	103.0	103.1	103.0	103.4	103.0	—	103.4	—	102.2	—	
Experiment II. Inoculated 2 cc. blood-liver mixture subcutaneously from rhesus No. 190, African strain, on Dec. 12																							
201	Control	—	103.1	103.9	104.4	Yellow Fever																	
		103.8	103.6	105.1	104.4	Dead																	
167	F. W.	102.9	103.6	103.0	103.1	103.4	102.4	103.0	103.4	103.0	103.6	102.6	102.9	102.7	102.9	103.6	103.9	103.9	104.0	103.7	103.4	103.8	
		103.8	103.3	103.1	103.3	102.9	102.9	103.1	103.2	103.5	103.6	102.8	—	103.4	—	103.8	104.0	104.0	103.9	103.9	103.6	—	
50	B. B.	—	102.2	103.0	102.6	102.8	103.2	103.4	102.9	103.1	103.3	102.0	101.9	102.0	102.8	102.5	102.7	103.0	103.6	102.9	102.7	—	
		102.0	102.5	102.9	102.2	103.0	103.5	103.5	103.0	103.2	103.6	102.0	—	102.2	—	102.4	102.9	103.4	102.5	—	—	—	
65	B. B.	—	102.8	103.6	103.0	102.9	103.1	102.9	103.1	102.9	103.4	102.4	102.8	102.6	102.1	102.3	102.2	102.8	103.0	102.8	103.0	—	
		103.2	103.1	103.2	103.6	103.4	102.9	103.0	102.9	103.2	103.3	102.2	—	102.8	—	102.4	102.5	102.9	102.2	—	—	—	
70	B. B.	—	102.0	103.0	103.0	102.5	102.8	102.4	103.0	102.9	103.0	102.3	103.4	103.1	103.0	102.9	103.3	103.4	102.8	102.5	102.8	—	
		102.8	102.2	103.4	102.9	102.5	102.6	102.8	103.4	102.9	103.3	103.1	—	103.2	—	103.0	103.5	103.0	102.2	—	—	—	
121	B. B.	—	102.9	102.8	102.9	103.2	103.0	102.7	103.1	102.4	102.1	102.2	103.0	103.1	103.2	102.8	102.6	102.4	102.6	102.8	102.5	—	
		102.6	102.2	102.8	103.0	103.4	102.9	103.0	102.6	102.5	103.0	103.2	—	103.4	—	102.9	102.8	102.5	102.6	102.8	102.5	—	

Dysentery												
Sacrificed when in extremis.												
114	B. B.	102 2	104 0	102 9	103 6	103 3	99 0	102 7	102 4	103 0	102 9	102 9
		102 2	104 0	102 9	103 6	103 3	98 4	102 7	102 4	103 0	102 9	102 9
108	B. B.	102 3	102 2	102 2	102 5	102 4	101 7	102 0	102 8	103 0	102 9	102 8
		102 3	102 2	102 2	102 5	102 4	101 6	102 0	102 8	103 0	102 9	102 8
130	F. W.	102 3	102 5	101 4	102 0	101 9	102 0	101 7	102 0	103 8	103 7	103 6
		102 3	102 5	101 4	102 0	101 9	102 0	101 7	102 0	103 8	103 7	103 6
131	B. B.	102 6	102 7	102 9	102 1	102 0	102 0	102 4	102 6	103 9	103 9	102 6
		102 6	102 7	102 9	102 1	102 0	102 0	102 4	102 6	103 9	103 9	102 6
135	F. W.	102 7	102 9	103 0	102 6	102 9	103 4	102 8	102 7	103 4	103 9	102 6
		102 7	102 9	103 0	102 6	102 9	103 4	102 8	102 7	103 4	103 9	102 6
140	B. B.	102 5	102 0	102 8	102 6	102 3	102 3	102 8	101 9	102 0	102 6	103 5
		102 5	102 0	102 8	102 6	102 3	102 3	102 8	101 9	102 0	102 6	103 5
147	B. B.	102 5	102 0	102 8	102 6	102 3	102 3	102 8	101 9	102 0	102 6	103 5
		102 5	102 0	102 8	102 6	102 3	102 3	102 8	101 9	102 0	102 6	103 5
Experiment III. Inoculated 2 cc. citrated blood subcutaneously from rhesus No. 209, African strain, on Jan. 5												
101	B. B.	102 8	102 0	103 1	103 0	103 4	103 6	102 9	103 3	103 1	102 4	102 8
		102 8	102 0	103 1	103 0	103 4	103 6	102 9	103 3	103 1	102 4	102 8
Experiment IV. Inoculated 2 cc. citrated blood subcutaneously from rhesus No. 229, African strain, on Jan. 7												
229	Control	103 0	103 8	105 7	104 9	102 4	102 4	103 6	103 0	103 6	103 2	103 0
		103 0	103 8	105 7	104 9	102 4	102 4	103 6	103 0	103 6	103 2	103 0
38	F. W.	102 9	103 4	104 1	104 0	101 1	103 9	101 3	103 4	103 0	102 5	102 4
		102 9	103 4	104 1	104 0	101 1	103 9	101 3	103 4	103 0	102 5	102 4
49	B. B.	103 4	103 2	103 4	103 3	103 4	103 5	103 6	103 4	103 2	103 8	103 4
		103 4	103 2	103 4	103 3	103 4	103 5	103 6	103 4	103 2	103 8	103 4
191	B. B.	103 0	102 6	102 0	103 6	103 9	104 4	104 0	103 6	103 4	103 2	103 0
		103 0	102 6	102 0	103 6	103 9	104 4	104 0	103 6	103 4	103 2	103 0
Experiment V. Inoculated 2 cc. citrated blood subcutaneously from rhesus No. 229, African strain, on Jan. 7												
231	Control	102 2	102 5	103 4	103 6	104 8	104 0	104 6	103 9	103 5	103 6	103 6
		102 2	102 5	103 4	103 6	104 8	104 0	104 6	103 9	103 5	103 6	103 6
41	J. V. O.	102 9	103 4	103 5	103 8	103 5	103 6	103 4	102 9	103 3	103 2	103 8
		102 9	103 4	103 5	103 8	103 5	103 6	103 4	102 9	103 3	103 2	103 8
79	B. B.	103 0	103 0	103 1	103 7	103 9	103 2	103 0	102 5	102 7	102 8	103 2
		103 0	103 0	103 1	103 7	103 9	103 2	103 0	102 5	102 7	102 8	103 2

TABLE II.

Previous History of Monkeys Used in Immunity Tests.

<i>M. rhesus</i> No.	Previous history
Experiment I	
190	Control. Not previously used
116	Inoculated with blood from S. R., Oct. 26. First fever Nov. 2; last fever Nov. 6; highest temperature 104.6°F.
118	Inoculated with blood from <i>rhesus</i> No. 104, Oct. 26. First fever Oct. 28; last fever Oct. 31; highest temperature 104.5°
122	Inoculated with blood from <i>rhesus</i> No. 100, Oct. 27. First fever Oct. 30; last fever Nov. 7; highest temperature 104.9°
125	Inoculated with blood from <i>rhesus</i> No. 118, Oct. 28, and from <i>rhesus</i> Nos. 111, 112, 114 and 118 on Oct. 29. First and last fever on Oct. 31; highest temperature 104.7°
31	Inoculated with blood from B. B., Sept. 2. First and last fever on Sept. 13; highest temperature 104.1°
Experiment II	
201	Control. Not previously used
167	Fed upon by mosquito batch No. 38, Dec. 1. First fever Dec. 4; last fever Dec. 6; highest temperature 105.3°
50	Inoculated with blood from <i>rhesus</i> No. 64, Oct. 2. First fever Oct. 4; last fever Oct. 7; highest temperature 104.6°. Used as an immune control for injection of tissues of No. 141 in Nov., without reaction
65	Inoculated with blood from <i>rhesus</i> No. 49, Sept. 28. First fever Oct. 4; last fever Oct. 5; highest temperature 104.8°
70	Inoculated with blood from <i>rhesus</i> No. 64, Oct. 2. First fever Oct. 4; last fever Oct. 14 (2nd rise); highest temperature 104.6°. Used as immune control for injection of blood-liver emulsion from Nos. 51, 152 and 155 in Nov., without reaction
121	Fed upon by mosquito batches Nos. 27 and 30, Oct. 27. First and last fever Nov. 1. Inoculated with blood of Nos. 146 and 129 on Nov. 13; temperature rose to 104.6°, Nov. 19
114	Inoculated with blood from <i>rhesus</i> No. 94, Oct. 24. First fever Oct. 28; last fever Oct. 31; highest temperature 105.0°
108	Inoculated with blood from <i>rhesus</i> Nos. 79 and 93, Oct. 23. First and last fever on Oct. 30; highest temperature 104.7°
130	Inoculated with blood from <i>rhesus</i> No. 123, Nov. 1. First and last fever Nov. 2; highest temperature 104.8°
131	Inoculated with blood-liver emulsion from <i>rhesus</i> No. 111, Nov. 1. First fever Nov. 2; last fever Nov. 6; highest temperature 105.4°. Inoculated with blood-liver emulsion from No. 101 in Nov., without reaction

TABLE II—*Concluded.*

<i>M. rhesus</i> No.	Previous history
Experiment II— <i>Concluded</i>	
135	Inoculated with blood from <i>rhesus</i> No. 130 on Nov. 2 and liver emulsion from No. 123 on Nov. 7. First fever Nov. 9; last fever Nov. 14; highest temperature 104.8°
140	Inoculated with blood from <i>rhesus</i> No. 136 on Nov. 5 and 6. First fever Nov. 9; last fever Nov. 10; highest temperature 104.8°
147	Inoculated with blood from <i>rhesus</i> Nos. 140 and 141, Nov. 9. First and last fever Nov. 10; highest temperature 104.8°
Experiment III	
229	Control. Not previously used
38	Inoculated with liver emulsion from monkey (series of Dr. Aragão) on Sept. 11. Fever from Sept. 19 to 22 and again from Sept. 30 to Oct. 2. Highest temperature 104.9°
49	Fourth passage monkey of B. B. strain. Inoculated Sept. 23. First and only fever Sept. 28; highest temperature 104.5°
191	Inoculated with blood-liver mixture from <i>rhesus</i> Nos. 170 and 180, also with immune serum on same date. Fever on 12th and 14th days; highest temperature 104.2°
Experiment IV	
231	Control. Not previously used
44	Inoculated with blood, liver, and spleen from <i>rhesus</i> No. 16, Oct. 5. Fever on Oct. 6, 8, 9 and 15; highest temperature 106.3°. Later immune to B. B. strain
79	Fed upon by mosquitoes Oct. 8. First fever Oct. 23, last fever Oct. 28; highest temperature 104.2°

in the control; another developed dysentery and was sacrificed when *in extremis* on the fifth day; six others showed a mild febrile reaction; fourteen had no fever.

Two of these animals had acquired their infections directly from human blood. Four Brazilian strains were represented. In point of time elapsed since the original attack of fever, one had gone slightly over three months and one only six days, the others for intermediate periods before the immunity test. Five animals had been used as immune controls for B. B. virus between the first attack and the inoculation with African virus.

There is no obvious explanation for the susceptibility of *rhesus* No. 118, which succumbed to the African virus. This animal apparently survived a typical, but only moderately severe, attack of the disease contracted by blood inoculation from *rhesus* No. 104. The latter was infected by mosquito bites and was a fairly severe case; the serum of *rhesus* No. 104 (taken 15 days after recovery) protected normal *rhesus* No. 191 against a large dose of B. B. virus, the control with non-immune serum, No. 192, being dead on the fifth day.

The origin of the Brazilian strains mentioned in Table I has been considered in a preceding paper (3). The African (Asibi) strain reached our hands on Dec. 1, 1928, and the *rhesus* (No. 175) inoculated on that date was found dead on Dec. 7. *M. rhesus* No. 185, whose blood was used in Experiment I of this series, was a subinoculation from No. 175 and also a fatal case. *M. rhesus* No. 190, third passage in our hands, was used for the inoculation of the animals in Experiment II. *M. rhesus* No. 209, the blood of which was used in Experiment III, was also a third passage, since it was infected from mosquitoes which had bitten No. 185. All fresh animals in first to fourth passages, as well as a fifth passage animal (subinoculation from No. 201), died with typical yellow fever. The control in Experiment IV, No. 231, was the first failure encountered. Even in this instance the onset of fever was perfectly definite and there is no doubt in our minds that the animal had yellow fever. The same dosage of virus from No. 229 killed *rhesus* No. 19.

Although Theiler and Sellards (2) are inclined to discount the value of temperature observations, we are disposed to regard them as valuable diagnostic aids when judiciously interpreted. It is perfectly true that one does not always detect a fever in fatal cases, even when temperatures are taken twice daily. It is equally true that many febrile reactions are not yellow fever. However, the indications are so often right that we consider well spent the time needed for this work.

CONCLUSIONS.

These experiments indicate that, although there are quantitative differences in virulence and minor differences in behavior, the African strain and the Brazilian strains of yellow fever virus tested are immunologically the same.

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ETIOLOGY OF OROYA FEVER.

XIV. THE INSECT VECTORS OF CARRION'S DISEASE.

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PLATES 45 TO 47.

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It is desirable to present the two parts of this investigation in a single paper, since they bear on each other so closely that to publish them separately will call for considerable repetition of statement. The origin of the studies which have led to the results here presented is to be found in the earlier papers of this series (1), and in the work of Townsend (2), who concerned himself especially with the insect vector of the disease embraced under the names of Oroya fever and verruga peruana. Since the observations given in this paper were completed after the death of Dr. Noguchi, who planned the experiments, we wish to state briefly the circumstances surrounding the investigation.

The earlier papers of the series established *Bartonella bacilliformis* as the bacterial incitant of Oroya fever (Carrion's disease) and verruga peruana. The etiology and much of the pathology of these manifestations of a single infectious disease were made clear by the experimental studies (Noguchi) carried out between 1925 and 1928. The essential fact which remained to be determined was the precise mode of infection in the two maladies for which the history and clinical observation had indicated an insect vector. Townsend's studies of the distribution of the disease and the nocturnal nature of its origin had led him to the decision that the vector belonged to the class of phlebotomi. Indeed, he had gone so far as to designate the vector as *Phlebotomus verrucarum*.

The cooperation of the International Health Division of the Rockefeller Foundation was secured in the field investigation of insects in the verruga zones in Peru. Various insect species were collected by one of us (Shannon), identified as far as possible on the grounds, the identifications being completed afterwards in the United States, and sent by ship to New York, where they were tested for infectivity on monkeys according to Dr. Noguchi's plans (Tilden and Tyler). The procedure employed in this testing was as follows:

The insects were collected without the use of chemicals and sealed alive in sterile tubes, which were either dry, or contained a piece of absorbent cotton moistened with sterile citrate solution (about half of each shipment was sent dry, the other half in moist condition). Collections were made near the time of sailing of the fast boats to New York, and shipments were placed in the steamer's refrigerator during transit.

The method of determining the presence of *Bartonella bacilliformis* in the insects was to inject a saline suspension of the crushed bodies intradermally, sometimes also intravenously, into monkeys (*Macacus rhesus*) and to make cultures of the blood at intervals of from one to four weeks later, irrespective of the occurrence of local lesions or fever. A given lot of insects was crushed in 0.9 per cent sodium chloride and injected intradermally into one or two sites on the shaved abdominal skin of two monkeys at least. Because of the differences in susceptibility of individual monkeys, duplicate tests were necessary.

The culture technique was the same as that used in earlier work on Carrion's disease (3). The blood was withdrawn from the monkey into an equal part of 2 per cent citrate in 0.9 per cent sodium chloride, and ascending dilutions in saline (1:10, 1:100, 1:1,000, 1:10,000, and 1:100,000) were inoculated into the semisolid leptospira medium (4) in amounts of 0.2 cc. One tube of the medium was inoculated with a drop of the undiluted citrated blood. The cultures were kept at 30°C. By the end of a two to three week period the positive cultures can usually be picked out by their macroscopic appearance, but even microscopic examination may fail to reveal a positive culture, and subculture is often desirable (5). The growth may be so slight in the initial culture as to be easily mistaken for the haze which develops in a tube of sterile medium after standing at 30°C.

Although *Bartonella bacilliformis* was detected only in the phlebotomi, it is desirable to state that other insects collected (Shannon) in the verruga zone were inoculated into *rhesus* monkeys in the manner of the phlebotomi and the blood cultures carried out in the same way as for the latter. In no instance was *Bartonella bacilliformis* isolated in these cultures. A list of the insects with which no results were obtained follows:

Ticks.—*Ornithodoros megnini* (on burros), *Argas* sp.? (on burros, birds, bats), Tick larvae (genus and species?) on lizard.

Mites.—*Tarsolomus* sp. (on ground), *Trombidium* n.sp. (on ground), *Geckobia* sp. (on lizard), *Geckobiella* sp. (on lizard and geckos).

Lice.—*Trichodectes ovis* (on sheep).

Fleas.—*Pulex irritans* (on man), *Ctenocephalus canis* (on cat, dog, and man), *Rhopalopsyllus* (on dog and guinea pig).

Bedbugs.—*Cimex lectularius*.

Mosquitoes.—*Anopheles pseudopunctipennis*, *Culex quinquefasciatus* (*fatigans*).

Buffalo gnats.—*Simulium escomeli*, *Simulium* sp. (on burros).

Midges.—*Forcipomyia utae*, *Forcipomyia townsendia*.

Muscidæ.—*Stomoxys calcitrans*.

Hippoboscidae.—*Meophagus ovinus* (on sheep).

Streblidae.—3 genera, one species each (on vampire bats).

Note should be made of the fact that Townsend (2) regarded the lizard as the natural reservoir of the incitant of verruga peruana, for the reason that he detected intracorpuseular bodies in the blood cells, which he identified with Barton's rods. Hence the red mites (*Trombidium*, *Tarsotomus*, *Geckobia*, *Geckobiella*), some of which were obtained from geckos (*Phyllodactylus reisi*), as well as the blood of two geckos, were injected into monkeys. Cultures prepared from the blood of these monkeys remained sterile.

Phlebotomi of Verrugas Cañon.

One of us (Shannon) spent from March to July, 1928, in the Rímac verruga zone, Peru. As many varieties of insects as possible were collected (6) from this zone and sent to The Rockefeller Institute in New York to be tested upon monkeys. It is desirable to state that Townsend was the first to implicate phlebotomus with the transmission of verruga peruana. His studies, conducted between 1912 and 1914, led him to decide, on ecological and experimental grounds, that a species of gnat, later called *Phlebotomus verrucarum* Townsend, was the vector of the disease.

Our studies revealed three species of *Phlebotomus* in the verruga zone. Two of the species had a wide and the third a limited distribution only in the zone. It is significant that of the three, only the two which occurred in considerable numbers were found on inoculation to yield *Bartonella bacilliformis*.

In this paper brief descriptions only will be given of the three species, based upon the characters of the males. All three species belong to the subgenus *Brumptomyia* (França and Parrot), which may be described as follows:

Basal segment of the upper appendage of the male terminalia with a well defined sub-basal tuft of hairs on the inner surface; the distal appendage either with four well developed spines, the fifth one weak, or with five strong spines; median appendage simple, without spines, lower appendage unarmed. The abdominal hairs are suberect to erect; length of the upper branch of upper forked vein longer than the petiole preceding the fork.

Ph. noguchii and *Ph. peruensis* are described here for the first time (Shannon).

Key to the Males.

1. Distal segment of upper appendage with four well developed spines, the fifth (an apical one) being very slender and hair-like; petiole of upper forked

The insects were collected without the use of chemicals and sealed alive in sterile tubes, which were either dry, or contained a piece of absorbent cotton moistened with sterile citrate solution (about half of each shipment was sent dry, the other half in moist condition). Collections were made near the time of sailing of the fast boats to New York, and shipments were placed in the steamer's refrigerator during transit.

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One of us (Shannon) spent from March to July, 1928, in the Rimac verruga zone, Peru. As many varieties of insects as possible were collected (6) from this zone and sent to The Rockefeller Institute in New York to be tested upon monkeys. It is desirable to state that Townsend was the first to implicate phlebotomus with the transmission of verruga peruana. His studies, conducted between 1912 and 1914, led him to decide, on ecological and experimental grounds, that a species of gnat, later called *Phlebotomus verrucarum* Townsend, was the vector of the disease.

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Ph. noguchii and *Ph. peruensis* are described here for the first time (Shannon).

Key to the Males.

1. Distal segment of upper appendage with four well developed spines, the fifth (an apical one) being very slender and hair-like; petiole of upper forked

cell slightly longer than that section of the first longitudinal vein which overlaps the upper branch of the second vein. . . . *Ph. verrucarum* Townsend.

2. Distal segment with five well developed spines, both apical ones being equally strong.

(a) Distal segment with two submedian spines, a third located slightly distal of the middle of the segment, the fourth and fifth forming an apical pair; petiole of the upper forked cell much longer than that section of the first vein which overlaps the upper branch of the second vein. (Type locality, Verrugas Cañon, Department of Lima, Peru)

Ph. noguchii Shannon.

(b) Distal segment with two submedian spines, a subapical one and an apical pair; that section of the first vein overlapping the upper branch of the second is distinctly longer than the length of the petiole of the upper forked cell. (Type locality, Matucana, Department of Lima, Peru)

Ph. peruensis Shannon.

The females of *Phlebotomus peruensis* can be separated from those of *verrucarum* and *noguchii* by differences in the arrangement of the wing veins, but definite characters have not yet been found whereby the females of *verrucarum* and *noguchii* may be positively identified. Approximate identification of the females which were sent to New York for bacteriological study was made on the basis of (1) average differences in size, *verrucarum* being in general smaller than *noguchii*, and (2) habitat. All males found in houses proved to be *verrucarum*, hence all females found in-doors were assumed to belong to this species. All the *noguchii* males were found out-of-doors, in excavations and natural cavities, where they were three times as numerous as *verrucarum* males.

Before leaving this description, it may be well to bring together certain accepted facts with reference to the epidemiology of Carrion's disease and certain known habits of the phlebotomi. It is admitted that the disease is contracted only in certain limited areas in Peru, and that infection, with possibly rare exceptions, takes place only at night. This infection may be acquired indoors or in localities remote from human habitations and at any time of the year.

With these facts in mind, it would seem to follow that the insect vector must be (a) common blood sucker of man; (b) restricted to the verruga zone; (c) nocturnal in habit; (d) capable of breeding in varied localities, so that adults, which have restricted flight, may be everywhere present, and (e) active throughout the year.

All these conditions are fulfilled by phlebotomi and not, as far as determined, by other insects of the verruga zones.

Finally, it may be recorded that we (Shannon and assistant) safely

spent from 2 to 5 nights a week for 4 months in the verruga zone after taking adequate precaution to protect ourselves from bites of phlebotomi.

Experiments with Phlebotomi.

Eighteen special lots of phlebotomi, prepared and shipped as described, were inoculated into monkeys (Tilden and Tyler). The material in each instance was introduced intradermally at several sites on the shaved skin of the abdomen, and was also applied to a scarified area of the abdominal skin. Occasionally an intravenous injection was also made.

Lots 1, 2, 9, and 14 were pooled, ground in a mortar with 0.9 per cent saline solution, and injected, Apr. 25, 1928, into two monkeys (*Macacus rhesus* I-3 and I-4).

Lot 1, collected Mar. 9, 1928, out-of-doors in Matucana. 3 females and 4 males of *Ph. noguchii*, *Ph. verrucarum*, and *Ph. peruensis*.

Lot 2, collected Mar. 9, 1928, out-of-doors in Matucana. 2 females, 18 males, *Phlebotomus*, chiefly *noguchii*.

Lot 9, *Ph. verrucarum* collected Mar. 20, 1928, in house in Verrugas Cañon. 40 females.

Lot 14, *Ph. verrucarum*, collected Mar. 26, 1928, in Verrugas Cañon. 24 females.

Lot 20. *Ph. noguchii*, with possibly a few *verrucarum* intermixed, collected Apr. 9, 1928, consisted of about 20 females. A saline suspension of the crushed insects was injected May 31, 1928, into two monkeys (*M. rhesus* I-7 and I-8). I-8 also received 1 cc. of the suspension intravenously.

Lots 27 and 28. *Ph. noguchii*, with possibly a few *verrucarum* intermixed, collected May 1 and May 8, 1928. 10 to 12 females. A saline suspension of the crushed insects was injected June 13, 1928, into two monkeys, *M. rhesus* I-17 and I-5.

Lots 29, 30, and 38. *Ph. verrucarum*, collected May 1 and May 8, 1928, 15 to 20 females. Saline suspension injected June 13, 1928, into *M. rhesus* I-16 and *M. rhesus* I-6.

Lots 40, 41, and 44. *Ph. verrucarum*, collected June 9, 11, and 18, 1928, both in houses and out-of-doors, in Verrugas Cañon. These lots comprised about 100 females. Saline suspension injected July 14, 1928, into *M. rhesus* I-26 and I-27. All the specimens which came in moist condition were covered with green mold.

Lots 39 and 45. *Ph. noguchii*, collected June 6 and June 19, 1928, out-of-doors in Verrugas Cañon. The number of insects was small (about 25 females), and the specimens which came in moist condition were covered with green mold. Saline suspension inoculated July 14, 1928, into *M. rhesus* I-28 and I-29.

Lots 42 and 46. *Ph. perucensis*, collected June 12 and 19, 1928, out-of-doors in Matucana. The specimens which came in moist condition were covered with green mold. 8 females. Saline suspension inoculated July 14, 1928, into *M. rhesus* I-30 and I-31.

Lot 43. *Ph. noguchii*, with possibly a few *verrucarum* intermixed, collected June 12, 1928, out-of-doors in Matucana. Saline suspension inoculated Aug. 13, 1928, into two monkeys, I-33 and I-34.

Lot 51. *Ph. verrucarum*, collected during the last week in July, 1928. 6 females. Saline suspension inoculated Aug. 13, 1928, into *M. rhesus* I-37.

Lot 54. *Ph. noguchii*, with possibly a few *verrucarum* intermixed, 8 to 10 females, collected during the last week in July out-of-doors in Verrugas Cañon. Saline suspension inoculated Aug. 14, 1928, into *M. rhesus* I-38 and I-39.

The first material inoculated, which contained all three species of *Phlebotomus* (Lots 1, 2, 9, and 14) yielded positive results.

Strain 1, from Lots 1, 2, 9, and 14.

M. rhesus I-3 and I-4, inoculated intradermally Apr. 25, 1928. No local lesions developed at the sites of inoculation. *M. rhesus* I-3 had a temperature of 104°F. on May 14, 19 days after inoculation, and blood was withdrawn on that day. *Bartonella bacilliformis* was obtained in culture from 1:10, 1:100, and 1:1,000 dilutions of the blood. The temperature reached 104°F. again several times, but blood culture was negative on May 29 and June 30. Blood was taken from *M. rhesus* I-4 at the same time as from I-3, but cultures remained negative.

Inoculation of Cultures from M. rhesus I-3.—*M. rhesus* I-14 and *M. rhesus* I-15 (Fig. 1) were inoculated June 5, 1928, with the 20 day culture of *Bartonella bacilliformis* obtained from the blood of *M. rhesus* I-3 and a 5-day subculture. The culture, which was, as usual, diluted with an equal part of saline for inoculation, was also applied to a scarified area on the abdominal skin. Tiny nodules were observed in *M. rhesus* 15 at the sites of intradermal inoculation on June 11 (16 days after inoculation), and 5 days later they were well developed, and one was excised¹ for examination and transfer. *Bartonella bacilliformis* was obtained from a 1:1,000 dilution of blood withdrawn June 16, and from a 1:100,000 dilution of the nodule suspension. By June 22 the scarified area showed small nodules. The photograph was taken 3 days later (Fig. 1). By June 28 the lesions had disappeared. *M. rhesus* I-14 had almost continuous high fever (104° to 106°F.) from June 8 to 28 and again from July 16 to 23, but no local lesions developed, and blood culture was negative 13, 31, and 55 days after inoculation.

The strain of *Bartonella bacilliformis* obtained from these first lots of phlebotomi was carried through two animal passages by direct transfer and has since been maintained by alternate generations in culture and

¹ All operations were carried out under ether anesthesia.

monkey. The usual course of verruga of moderate severity (7) has been observed in the animals (Fig. 8), with the exception of one monkey of the first passage (*M. rhesus* I-1), which had an unusually severe cutaneous reaction (Figs. 6, 7), not unlike that which had been induced in one of the monkeys (*M. rhesus* 18) of an early experiment (Noguchi (8)). This animal was acutely ill over a period of two weeks but recovered.

Histological study of the nodular tissue from I-15 and I-1, made by Dr. Henry R. Muller, shows the characteristic proliferation of endothelial cells and the formation of new capillaries. *Bartonella bacilliformis* was detected in some instances within the endothelial cells.

The second lot of phlebotomi tested, which probably consisted chiefly of *Ph. noguchii* but may have contained a few *verrucarum*, also yielded a strain of *Bartonella bacilliformis*.

Strain 2 from Lot 20.

M. rhesus I-7 and I-8 were inoculated May 31, 1928, intradermally, and I-8 received 1 cc. of the suspension in the left saphenous vein. The animals were bled on June 11 and again on June 30. The blood of I-8 yielded cultures of *Bartonella bacilliformis* in 1:100,000 dilution on June 11 and in 1:100 dilution on June 30; no cultures were obtained from the blood of I-7. Neither animal developed lesions at the sites of inoculation, but I-8 had almost continuous fever (104° to 105.2°F.) from June 4 to June 28.

Inoculation of Culture from M. rhesus I-8.—*M. rhesus* I-22 and I-23 were inoculated on June 22, 1928, intradermally, with a 10-day-old culture from the blood of *M. rhesus* I-8. The culture was also applied to a scarified area on the abdomen. The nodules showed at the sites of intradermal inoculation after about 10 days, and blood withdrawn after 2 weeks yielded cultures of *Bartonella bacilliformis* in 1:10,000 dilution. The nodule excised¹ on July 6 for examination and transfer yielded cultures in a 1:100 dilution. The lesions were considerably larger and more extensive in I-23 (Fig. 3), and the edema of the abdominal wall developed early and became marked. Regression of the lesions began about four weeks after inoculation and within four weeks recovery was practically complete. The course of disease was almost afebrile in both animals.

Further inoculations, first with nodular tissue from I-22 and I-23, and later with cultures from the blood of passage animals, showed that this strain of *Bartonella bacilliformis*, like Strain 1, was moderately virulent, inducing pronounced local lesions and moderate anemia.

One animal of the series died in 33 days, after an afebrile course of disease, during which moderately severe anemia had been observed.

Lots 27 and 28 (chiefly *Ph. noguchii*) and Lots 29, 30, and 38 (*Ph. verrucarum*) yielded negative results, as did also Lots 40, 41, 44, and 51 (*Ph. verrucarum*), Lots 42 and 46 (*Ph. perucensis*), and Lot 43 (*Ph. noguchii*).

From Lots 39 and 45, which probably consisted of *Ph. noguchii* alone, a third strain of *Bartonella bacilliformis* was obtained.

Strain 3, from Lots 39 and 45.

M. rhesus I-28 and I-29 were inoculated intradermally on July 14, 1928. Monkey I-28 showed no fever at any time, and blood cultures were negative 1, 2, and 3 weeks after inoculation. Monkey I-29 had fever (104.2° to 105.2°F.) 3 days after inoculation, which continued for a week with one day of remission. The blood yielded cultures of *Bartonella bacilliformis* in a dilution of 1:100 on four occasions, 7, 13, 23, and 31 days after inoculation, but there was no reaction at the sites of injection.

Inoculation of Cultures from I-29.—*M. rhesus* I-44 was inoculated intradermally and by scarification on Aug. 16, 1928, with a culture 14 days old from the blood of *M. rhesus* I-29. 1 cc. of the culture was also injected into the saphenous vein. Nodules developed in 2 weeks (Figs. 5 and 9), and the scarified area presented the characteristic miliary eruption. The blood was positive in a dilution of 1:10,000 at this time. From Sept. 7 to 19 there was marked fever (104° to 105.2°F.).

M. rhesus I-45 was inoculated at the same time and in the same manner as I-44. The intradermal nodules attained a diameter of only 0.5 cm., and the blood was positive in a dilution of 1:100. Fever existed (104.2° to 105.4°F.) from Sept. 10 to 13, was followed by two days of subnormal temperature, and death of animal on Sept. 16. Autopsy (Dr. Muller) revealed nothing abnormal except in the spleen, which contained numerous pale areas 2 to 3 mm. in diameter. Film preparations were negative for tubercle bacilli, and microscopic examination disclosed infarcts such as are found in the spleen in human (9) cases of Oroya fever, and in cases of the experimental disease (10).

Later passage of Strain 3 produced local lesions of very large size (2 to 3 cm. in diameter), but no unusual systemic effects.

Lot 54, which consisted chiefly, perhaps wholly, of *Ph. noguchii*, also yielded *Bartonella bacilliformis*.

Strain 4, from Lot 54.

M. rhesus I-38 was injected intradermally on Aug. 14, 1928, and intravenously (1 cc. of the saline suspension into the left saphenous vein). From Aug. 22 to

Aug. 29 the temperature was 104°F., but blood culture made Aug. 28 was negative. It was also negative on Sept. 10, but blood taken on Sept. 25, when the temperature was 104.2°F. yielded *Bartonella bacilliformis* in 1:10 and 1:100 dilutions after 13 days incubation. The intradermal mixtures produced no lesions. *M. rhesus* I-39, inoculated at the same time as I-38, and with the same material, showed a rise of temperature (104.2° to 104.8°F.) on three occasions, but blood cultures made on Aug. 28, Sept. 10, and Sept. 25 were negative.

Inoculation of Cultures from I-38.—*M. rhesus* I-58 was inoculated on Oct. 10, 1928, with 15-day culture from the blood of *M. rhesus* I-38. Small nodules appeared at the sites of intradermal injection after 7 days and were well advanced after 16 days (Fig. 5). The abdominal wall became oedematous and the area of scarification showed miliary nodules in addition to which three or four small eruptions arose outside the inoculated areas. Blood culture was positive in dilutions up to 1:10,000, 12 days after inoculation. The animal died on the 18th day, when the local lesions were still actively progressing. Histological examination of tissues by Dr. Muller revealed the characteristic zonal necrosis around the central vein in the liver, with extensive invasion by polymorphonuclear leucocytes. The spleen showed no lesions. The various skin nodules were histologically characteristic of verruga in the monkey.

Further inoculations with cultures of Strain 4 yielded similar results. In one animal (*M. rhesus* S-7) the local lesions reached large size.

The results of the inoculations are summarized in Tables I to V.

Exposure of Monkeys to Bites of Phlebotomi.

Six *rhesus* monkeys were exposed (Shannon) for several weeks to natural infection, three in an excavation in Verrugas Cañon, where *Ph. noguchii* was fairly common, and three in a house where *Ph. verrucarum* was abundant. These animals were brought to The Rockefeller Institute on Aug. 13. Blood withdrawn on three occasions failed to yield cultures of *Bartonella bacilliformis*, and only one of the animals failed to respond to subsequent inoculation of virulent cultures or passage virus. The result therefore was regarded as negative.

Immunity.

Seven of the monkeys which had developed verrucous lesions and blood infection with *Bartonella bacilliformis* following inoculation with the *Phlebotomus* strains and in which the lesions had regressed, were subsequently tested for immunity by reinoculation. Similar immunity tests were made on three monkeys which had received crushed

TABLE I.
Inoculations of Crushed Phlebotomi.

<i>M. rhesus</i> No.	Date 1928	Lot No.	Method of inoculation	Local lesions	Blood culture
I-3	Apr. 25	1, 2, 9, 14 <i>verrucarum</i> <i>noguchii</i> <i>peruensis</i>	Multiple intradermal Scarification	—	+
I-4	Same	Same	Same	—	—
I-7	May 31	20 <i>noguchii</i> (few <i>ver-</i> <i>rucarum</i> ?)	Multiple intradermal Scarification	—	—
I-8	Same	Same	Same, also intravenous	—	+
I-16	June 13	29, 30, 38 <i>verrucarum</i>	Multiple intradermal Scarification	—	—
I-6	Same	Same	Same	—	—
I-17	Same	27, 28 <i>noguchii</i> (few <i>ver-</i> <i>rucarum</i> ?)	Same	—	—
I-5	Same	Same	Same	—	—
I-26	July 14	40, 41, 44 <i>verrucarum</i>	Same	—	—
I-27	Same	Same	Same	—	—
I-28	Same	39, 45 <i>noguchii</i>	Same	—	—
I-29	Same	Same	Same	—	+
I-30	Same	42, 46 <i>peruensis</i>	Same	—	—
I-31	Same	Same	Same	—	—
I-33	Aug. 13	43 <i>noguchii</i> (few <i>ver-</i> <i>rucarum</i> ?)	Same	—	—
I-34	Same	Same	Same	—	—
I-37	Same	51 <i>verrucarum</i>	Same	—	—
I-38	Same	54 <i>noguchii</i> (few <i>ver-</i> <i>rucarum</i> ?)	Same, also intravenous	—	+
I-39	Same	Same	Same	—	—

TABLE II.

Strain 1, from Lots 1, 2, 9, 14 (Ph. verrucarum, Ph. noguchii, Ph. perusis).

<i>M. rhesus</i> No.	Date 1928	Material inoculated	Mode of inoculation	Local lesions	Blood culture
I-14	June 5	Culture from I-3	Intradermal Scarification	—	—
I-15	Same	Same	Same	+++	+
First passage					
I-18	June 16	Nodule susp. I-15	Same	++++	+
I-19	Same	Same	Same	++++	+
I-1	Same	Same	Same	++++	+
Second passage					
I-12	July 6	Nodule susp. I-1	Same	++++	+
I-13	Same	Same	Same	+++	+
Third passage (via culture)					
I-40	Aug. 16	14-day culture I-12	Same, also intra- venous	++++	+
I-41	Same	Same	Same	++++	+

TABLE III.

Strain 2 from Lot 20 (Ph. noguchii—few verrucarum?).

<i>M. rhesus</i> No.	Date 1928	Material inoculated	Mode of inoculation	Local lesions	Blood culture
I-22	June 22	10-day culture from I-8	Intradermal Scarification	++++	+
I-23	Same	Same	Same	++++	+
First passage					
I-24	July 6	Nodule susp. I-23	Same	++++	+
I-25	Same	Same	Same	+++	+
Second passage (via culture)					
I-42	Aug. 16	14-day culture from I-24	Same, also intrave- nous	++++	+
I-43	Same	Same	Same	++++	+

TABLE IV.
Strain 3 from Lots 39 and 45 (Ph. noguchii).

<i>M. rhesus</i> No.	Date 1928	Material inoculated	Mode of inoculation	Local lesions	Blood culture
I-44	Aug. 16	14-day culture from I-29	Intradermal Scarification	++++	+
I-45	Same	Same	Intravenous Same	++++	+
First passage					
I-55	Sept. 13	Nodule susp. I-44	Intradermal Scarification	+++	+
I-56	Same	Same	Same	++	-
I-34	Oct. 22	20-day culture from I-45	Same	+++	+
Second passage (via culture)					
S-6	Dec. 15	25-day culture from I-34	Same	++++	+

TABLE V.
Strain 4 from Lot 54 (Ph. noguchii—few verrucarum?).

<i>M. rhesus</i> No.	Date 1928	Material inoculated	Mode of inoculation	Local lesions	Blood culture
I-58	Oct. 10	15-day culture from I-38	Intradermal Scarification	+++	+
First passage (via culture)					
I-17	Nov. 5	14-day culture from I-58	Same	++++	+
Second passage (via culture)					
S-7	Dec. 15	18-day culture from I-17	Same	++++	+

TABLE VI.
Immunity Tests.

<i>M. rhesus</i> No.	First inoculation				Immunity test			
	Date, 1928	Material inoculated	Local lesions	Blood culture	Date, 1928	Material inoculated	Local lesions	Blood culture
I-18	June 16	Nodule susp. I-15 (Str. 1)	++++	+	Sept. 13	Nodule susp. I-41 (Str. 1)	—	—
I-13	July 6	Same	++++	+	Same	Same	—	—
I-3	Apr. 25	Phlebotomi Lots 1, 2, 9, 14	—	+	Same	Same	++++	—
I-53 Control					Same	Same	++++	+
I-8	May 31	Phlebotomi Lot 20	—	+	Sept. 13	Nodule susp. I-43 (Str. 2)	++++	+
I-25	July 6	Nodule susp. I-23 (Str. 2)	++++	+	Same	Same	—	—
I-23	June 22	Blood culture I-8 (Str. 2)	++++	+	Same	Same	—	—
I-11	July 6	Nodule susp. P. 5*	++++	+	Same	Same	—	—
286	June 1	Culture P. 5*	++++	+	Same	Same	—	—
I-54 Control					Same	Same	+++	+
I-1	June 16	Nodule susp. I-15 (Str. 1)	++++	+	Sept. 26	Culture P. 5*	—	—
I-19	June 16	Same	++++	+	Same	Same	—	—
I-29	July 14	Phlebotomi Lots 39, 45	—	+	Same	Same	+++	+
I-57 Control					Same	Same	+++	+

* Noguchi, H., *J. Exp. Med.*, 1927, xlv, 175.

phlebotomi without developing skin lesions which reacted as do previously untreated animals. Table VI summarizes the results.

Morphology.

No morphological or cultural differences could be detected between the *Phlebotomus* strains and the human strains of *Bartonella bacilliformis*. Cultures seven days old of the four strains, grown on horse blood agar slants, were stained to bring out the unipolar flagella (one to four) which are characteristic of *Bartonella bacilliformis* (Figs. 11, 13, 15, 17), the films being made on the same slide, in order that the stained preparations might be comparable. Cultures of the same age but grown on leptospira medium were used for similar comparative preparations which were stained by Gram's method, with fuchsin as the counterstain (Figs. 10, 12, 14, 16).

SUMMARY AND CONCLUSIONS.

With a view to determining the mode of infection in Carrion's disease, a study of the blood-sucking insects found in the districts of Peru where the disease prevails has been carried out, through the co-operation of The Rockefeller Institute and the Rockefeller Foundation. The material studied included ticks, mites, midges, lice, fleas, bedbugs, mosquitoes, buffalo gnats, horse-flies, "sheep ticks," 3 species of Streblidae, and 3 species of *Phlebotomus*, including *Phlebotomus verrucarum* Townsend and two new species which have been named *Phlebotomus noguchii* and *Phlebotomus perucensis*. The insects were collected without the use of chemicals, were prepared for transportation in such a manner as to prevent drying, and were shipped under conditions of refrigeration to New York, where they were inoculated into monkeys. The plan followed was to inject saline suspensions of the crushed insects intradermally into *rhesus* monkeys and to make cultures of the blood of the animals at intervals of 1 to 6 weeks after inoculation.

The only class of insects in which the presence of *Bartonella bacilliformis* could be detected were phlebotomi. No cutaneous lesions were induced in monkeys injected with the crushed insects, but in the case of four different lots of phlebotomi the blood of the animals so injected yielded cultures of *Bartonella bacilliformis* which produced typical verrucous lesions on inoculation into other monkeys.

The morphology and cultural characteristics of the *Bartonella* strains obtained from phlebotomi proved identical with those of strains

isolated from human blood and skin lesions. Monkeys which had recovered from infection with the phlebotomus strains resisted inoculation with a human strain of *Bartonella bacilliformis*, and, conversely, monkeys which had passed through an infection induced by the human strain resisted inoculation with the strains obtained from phlebotomi.

The experimental observations described in this paper lead us to conclude that certain phlebotomi act as insect vectors of Oroya fever and verruga peruana. The phlebotomi which have been shown quite certainly to carry the *Bartonella bacilliformis* are those of the species *Phlebotomus noguchii*. *Phlebotomus verrucarum* is also probably a vector, while *Phlebotomus peruensis* remains doubtful in this respect.

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EXPLANATION OF PLATES.

PLATE 45.

FIG. 1. Cutaneous lesions induced in *M. rhesus* I-15 by Phlebotomus Strain 1. Photograph taken 20 days after inoculation. One intradermal nodule had been excised 2 days previously.

FIG. 2. The appearance of the lesions in *M. rhesus* I-1, a Strain 1 first passage animal, 18 days after inoculation. The sacrificed area already shows characteristic minute nodules. All the lesions reached considerable size (Figs. 6 and 7).

FIG. 3. Strain 2. Early culture lesions (two weeks after inoculation) in *M. rhesus* I-23.

FIG. 4. *M. rhesus* I-44, 21 days after inoculation with cultures of Strain 3 from Lots 39 and 45 of *Ph. noguchii*.

FIG. 5. *M. rhesus* I-58, 16 days after inoculation with Strain 4 phlebotomus cultures. The eruption was more general and the edema extensive in this animal. Death occurred 3 days after the photograph was made.

PLATE 46.

FIGS. 6 AND 7. Late lesions in *M. rhesus* I-1 (Strain 1) as they appeared 31 days after inoculation. The most pronounced lesion occurred at the scarification site (center).

FIG. 8. *M. rhesus* I-19, 29 days after inoculation in the same way and at the same time as *M. rhesus* 1.

FIG. 9. *M. rhesus* I-44, 21 days after inoculation with cultures of *Phlebotomus noguchii* Strain 3.

PLATE 47.

Magnification $\times 1,000$.

FIG. 10. Phlebotomus Strain 1, from Lots 1, 2, 9, 14. Gram's stain, counterstained with saturated alcoholic solution of fuchsin.

FIG. 11. Same, stained for flagella, by a combination of Zettnow's mordant and Fontana's ammoniac silver solution.

FIG. 12. Phlebotomus Strain 2, from Lot 20. Gram's stain, counterstained with fuchsin.

FIG. 13. Same, Zettnow-Fontana flagella stain.

FIG. 14. Phlebotomus Strain 3, from Lots 39 and 45. Gram's stain, counterstained with fuchsin.

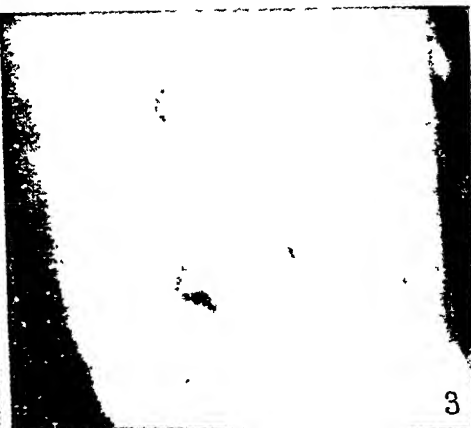
FIG. 15. Same, Zettnow-Fontana flagella stain.

FIG. 16. Phlebotomus Strain 4, from Lot 54. Gram's stain, counterstained with fuchsin.

FIG. 17. Same, Zettnow-Fontana flagella stain.



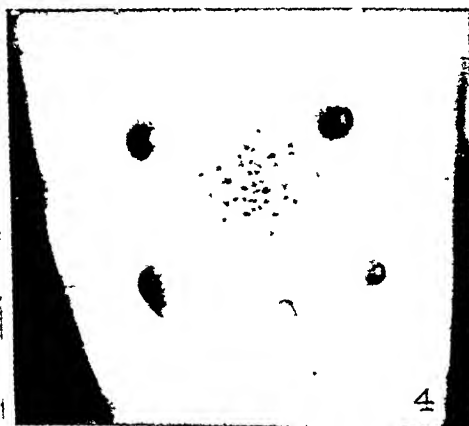
M. rhesus I-15. Strain 1.
20 days after inoculation.



M. rhesus I-23. Strain 2.
14 days after inoculation.



M. rhesus I-1. Strain 1
18 days after inoculation.



M. rhesus I-44. Strain 3.
21 days after inoculation



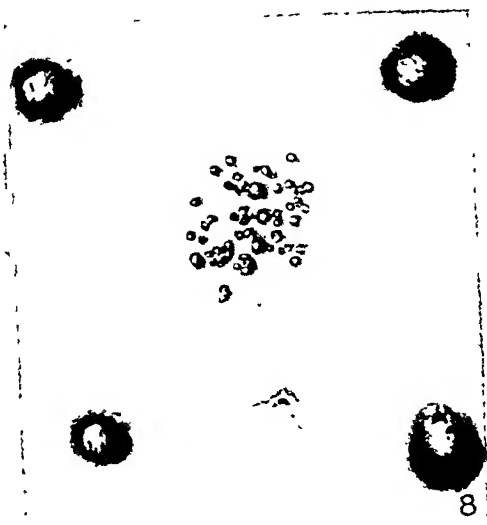
M. rhesus I-58. Strain 4.
16 days after inoculation.





M. rhesus I-1, Strain 1,
31 days after inoculation

6



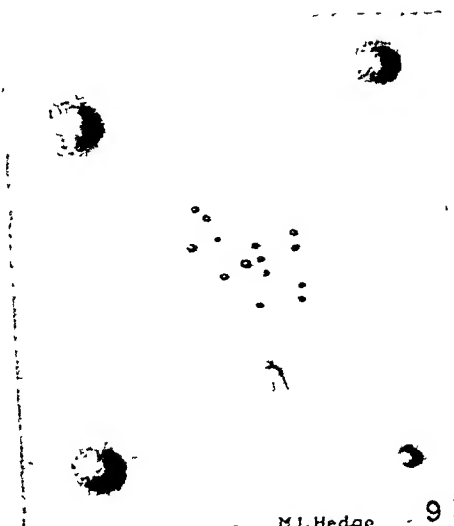
M. rhesus I-19, Strain 1,
29 days after inoculation.
Scarified area in center.

8



M. rhesus I-1, Strain 1, 31 days after
inoculation. The largest lesion arose
on the area inoculated by scarifica-
tion (center)

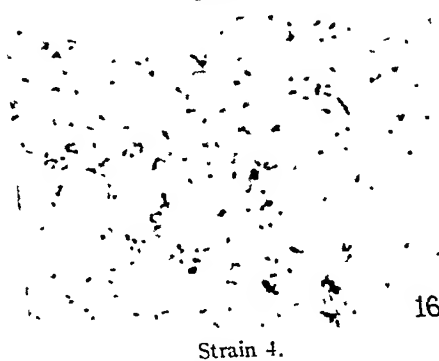
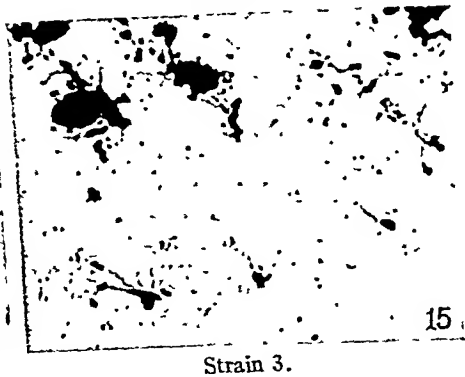
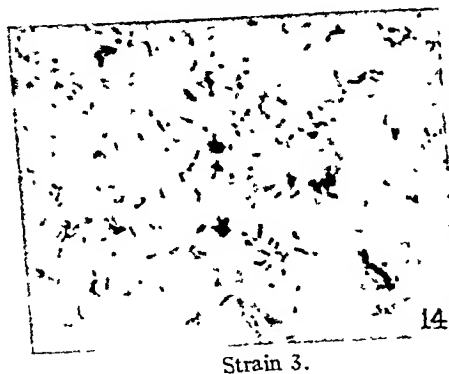
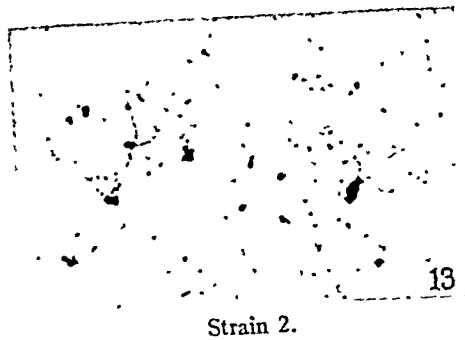
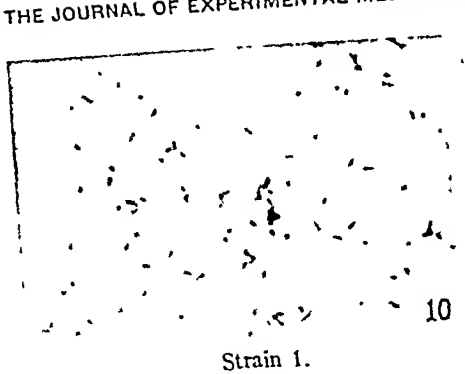
7



M. rhesus I-44, Strain 3,
21 days after inoculation.
Scarified area in center

M.L. Hedge

9



Gram, counterstained with fuchsin.

Zettnow-Fontana combination stain.

× 1,000.

(Noguchi *et al.*: Etiology of Oroya fever. XIV.)

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Edited by

W. J. GROZIER

JOHN H. NORTHROP

W. J. V. OSTERHOUT.

The Journal of General Physiology is devoted to the explanation of life phenomena on the basis of the physical and chemical constitution of living matter.

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BLOOD CALCIUM DISTRIBUTION IN ANAPHYLAXIS IN THE GUINEA PIG.

BY HERMAN BROWN AND SUSAN GRIFFITH RAMSDELL.
(From the Research Institute of Cutaneous Medicine, Philadelphia.)

(Received for publication, January 24, 1929.)

The biological importance of calcium has, of course, long been known; but it has only been comparatively recently, through the stimulus afforded by the findings of the close relationship between certain pathological conditions and disturbed calcium metabolism, that the element has been so extensively studied. The effect of calcium on tissue permeability and irritability, and the rôle attributed to it in blood coagulation is of special interest in the study of anaphylaxis; and the work to be reported here is a study of the variations in the total calcium and in the diffusible and non-diffusible fractions which occur in the anaphylactic guinea pig.

While many determinations have been made of total serum calcium, usually with findings of a constant or only slightly diminished value (1-3), a review of the literature yields no report on diffusible calcium in anaphylactic shock. Blum, Delaville and Caulaert (4) found the diffusible calcium in peptone shock in one dog increased from 5.7 to 7.5 mg. per 100 cc.; and in the rabbit, in which the manifestations of shock were weak, two experiments gave nearly double the values for the diffusible fraction over that found in the control animal. No such study is to be found for the guinea pig, in which, of all the experimental animals, anaphylactic shock is to be demonstrated most uniformly and definitely.

Technic.

For this study 66 guinea pigs were used, including the normal controls. The shocked animals were prepared by direct sensitization with human or horse serum, or passively by the transfer (48 hours) with an anti-horse serum (rabbit). Since the size of the animal precluded preshock bleedings, subsensitized animals, for controls, were injected with horse serum and then killed after varying intervals corresponding to those at which the shocked animals were bled.

In all the groups it was necessary to use the pooled bloods of from 2 to 4 animals, depending upon their size, in order to secure a sufficient sample of serum for analysis. Care was taken, in all cases, that the animals in each group showed the same type of reaction, and that the bleedings were made at the same intervals after injections.

Calcium determinations were made according to Tisdall's modification (5) of Kramer and Tisdall's method (6). The method of Moritz (7) as modified by Updegraff, Greenberg and Clark (8) was used in obtaining the diffusible calcium findings. As a criterion of a proper permeability of the collodion bags, we accepted only those results in which not less than 1 cc. of fluid passed through the membrane and which, of course, gave no tests (biuret) for protein in the diffusate. Nearly all results given in the table are averages of two determinations and all the results were actual ones, *i.e.*, the non-diffusible calcium was not calculated by difference. This we believe to be an added check on the results, for though the possibility has been suggested (8) that some Ca may be lost in the membrane itself, an inspection of the tables will indicate that the sum of the diffusible and non-diffusible calcium is, in general, rather more, instead of less, than the total calcium, indicating that such losses are quite negligible and are more than counterbalanced by the errors inherent in the calcium determination itself.

DISCUSSION.

In the table are listed the results of determinations for total serum calcium, the diffusible and non-diffusible, and the percentage of these of the total calcium. All the figures are placed in one table to facilitate comparison of the normal findings with those for the animals which were subsensitized and did not show shock symptoms and those which showed a marked degree of anaphylaxis.

An inspection of the table indicates, in the first place, that the total serum calcium is quite constant in the guinea pig even in cases of severe shock. However, much work has been done in recent years to show that the biological activity of calcium really depends upon the various forms in which it may exist. Since the discovery of Rona and Takahashi (9) that serum calcium exists in diffusible and non-diffusible form, much evidence has accumulated (10-12) to indicate that the non-diffusible fraction is in the form of a protein compound. This explanation has been rejected by Cameron and Moorhouse (13) and, though it is not the purpose of this paper to discuss the merits of these theories, we should like to point out, that, at present, it appears to be accepted that the non-diffusible serum calcium is in the form of a protein complex. Moreover, the total serum calcium determination

may be a poor index of what is really happening, for a disturbance of this calcium-protein complex may occur with resulting marked

TABLE I.

Blood Calcium Distribution in the Normal and Shocked Guinea Pig.

Experiment No.	Total Ca	Diffusible Ca		Non-diffusible Ca		Remarks
		Mg. per 100 cc.	Per cent of total	Mg. per 100 cc.	Per cent of total	
	<i>mg. per 100 cc.</i>					
1	10.2	5.4	53	4.5	44	Normal animals
2	8.6	4.6	54	4.0	47	Normal animals
3	10.2	5.9	58	4.0	44	Normal animals
4	8.6	4.7	55	3.6	42	Normal animals
5	9.6	6.5	67	3.2	33	Normal animals
6	10.1	5.3	52	5.0	49	Doubtful shock 20 min. after antigen injection
7	9.4	5.0	53	4.2	45	Negative shock. Bled 20 min. after injection
8	9.4	5.1	54	4.2	45	Doubtful shock. Bled 20 min. after injection
9	8.8	5.2	59	3.5	40	Negative shock. Passive sensitization
10	8.4	4.9	58	3.6	43	Maximum shock; bled at prostration
11	10.0	7.1	71	3.2	32	Maximum shock; bled at prostration
12	9.3	5.9	63	3.2	34	Maximum shock; bled at prostration
13	9.2	6.6	72	2.7	30	Maximum shock; bled at prostration
14	9.7	6.2	64	3.6	37	Maximum shock; bled at prostration; passive sensitization
15	9.3	6.7	72	3.1	33	Immediate shock; bled on appearance of clonic seizures; lungs maximum
16	9.6	5.7	59	4.1	43	Immediate shock; bled on appearance of clonic seizures; lungs maximum
17	10.1	7.3	72	3.0	30	Immediate shock; bled on appearance of clonic seizures; lungs maximum
18	10.0	7.3	73	2.7	27	Histamine, 1 cc. 1/5000 dilution; immediate violent shock; bled at prostration

physiological effects, but without altering the total serum calcium. A striking example of this has recently been pointed out by Shelling and

Maslow (14) who demonstrated that, following citrate additions to serum, either *in vitro* or *in vivo*, practically the entire calcium-protein combination is destroyed, all the calcium becoming diffusible, without, however, altering the total serum calcium. In order to determine if the reaction in the guinea pig following citrate injection resembled anaphylaxis, Shelling and Maslow's procedure was followed in one set of animals: calcium determinations (not listed in the tables) indicated that over 80 per cent of the guinea pig serum calcium became diffusible. Following the citrate injections violent immediate reactions were elicited; these, however, did not in any way simulate those characteristic of anaphylactic shock.

The findings for diffusible calcium, in terms of percentage of total calcium, are shown in the table to vary from 53 to 67 with an average of 55 for the normal animals; for the subsensitized which did not manifest typical symptoms upon reinfection, the variations were from 52 to 59 with an average of 54.5; while in the shocked animals a minimum value of 58 and a maximum of 72, with an average of 66, were found. Experiments 10 and 16 of the shocked animals gave values which may be considered "high" normal, while the results of the other experiments indicated a very definite increase in diffusible calcium with a corresponding decrease in the non-diffusible or "protein-complex" calcium.

There is then no change in this form of calcium incident to the treatment of the animal with a foreign serum, but a decided increase when the animals manifest shock. This increase is apparently not affected by the time interval at which the animal is bled, once symptoms of acute shock are established.

That the calcium change is not peculiar to anaphylactic shock is evidenced by the experiment with citrate injections and with histamine, resulting in the highest figure found for diffusible calcium. But in this last type of reaction, the coagulability of the blood is known not to be affected. This change in calcium ion content of the blood in anaphylactic shock is then not of necessity directly related to the phenomenon of a lessened coagulation time. This last has, indeed, been adequately accounted for by experiments of Weil (15) which demonstrated that the lessened coagulation time could be attributed to a liver product thrown into circulation following contact with the

antigen. From the results found for the non-diffusible fraction, it may be inferred that some change occurs in the protein of the serum whereby its normal combination with calcium is disturbed; but the data at hand furnish no basis for any conclusions as to the rôle this change plays in the basic mechanism of the phenomenon of shock.

CONCLUSION.

The results above reported for total calcium and the membrane-diffusible fraction in the serum of the guinea pig, taken at various intervals during anaphylactic shock, confirm the findings of previous workers that the total calcium is essentially unchanged. There is, however, the further finding that the diffusible fraction is considerably increased over that found for the animal similarly treated but not manifesting characteristic symptoms.

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OXIDATION-REDUCTION EQUILIBRIA IN BIOLOGICAL SYSTEMS.

II. POTENTIALS OF AEROBIC CULTURES OF *B. TYPHOSUS*.

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Gillespie (1) was the first to observe that an indifferent metallic electrode placed in a culture of bacteria manifests a negative drift in potential. Potter (2) had found previously that an electrode in an inoculated portion of culture medium is negative to an electrode in an uninoculated portion of the same medium, when the two portions are separated by a porous diaphragm. Clark (3) and his collaborators have extended the observations of Gillespie and have aroused a widespread interest in the biochemical processes involved in the establishment of reduction potentials.

The hopes of the earlier investigators that the determination of reduction potentials would provide a measure of the intensity of oxidation or reduction in biological systems cannot yet be realized, since difficulties are apparent in the interpretation of such potentials in strict accordance with the theory of oxidation-reduction equilibrium as it has been developed for inorganic or relatively simple organic substances. The substances responsible for the observed potentials of biological complexes have not been identified; there is much to suggest, however, that the sulphydryl bodies are involved. The work of Dixon (5) and Dixon and Quastel (4) and the recent careful study of Michaelis and Flexner (6) indicate that these bodies do not constitute a simple reversible oxidation-reduction system, as would be required for strict application of the theory of reduction potentials, although their solutions in the absence of oxygen yield definite potentials which vary with the C_H and the concentration of the reduced form of the substance. These substances are, therefore, electromotively active and the combined form glutathione is so generally present in biological preparations that it must be concerned in the production of electrode potentials. These and other electromotively active bodies may also actually intermediate the reduction potentials, acting as catalysts for the oxidation or reduction of organic constituents which are not themselves electromotively active. There is, however, at the present time no evidence that the reactions which may be catalyzed by such substances are subject to definition in terms of intensity values. Further data from biological systems

must be accumulated before a formulation of the mechanisms involved can be attempted.

No satisfactory explanation has been offered for the part played by molecular oxygen in the development of biological reduction potentials, although all investigators have noted that reduction potentials of considerable intensity are attained only when oxygen is removed from the system. This may be accomplished by deaeration by a stream of nitrogen or by a spontaneous oxygen consumption which may take place as in sterile bouillon (7) even when the system contains only inanimate materials. Before such anaerobic conditions are attained the potentials in any biological system are distinctly more positive than when oxygen is absent, and it is not possible to decide to what extent the electrode under these conditions acts simply as an oxygen electrode or measures the actual oxidation-reduction forces which prevail in the system or indeed to decide whether or not these two possible mechanisms are properly to be contrasted.

Limitations thus surround the investigation of biological potentials and the significance which may be attached to the results.

In the first paper (7) of this series the reduction potentials of sterile culture bouillon were examined, and it was found that sterile bouillon in the absence of oxygen yields rather definite and characteristic potential values. Although the significance of these values is limited it seemed possible that they might serve as comparison values in a study of the potentials of growing cultures. The subject of the present paper is the relation of the potentials of sterile bouillon to those of living cultures of *B. typhosus* in the same medium. It was the hope that such an investigation would throw light upon the reducing activities of bacteria and upon the mechanism by which oxidation-reduction potentials are produced.

Technic.

The arrangement of the electrode cell and the method of potential measurement was that described in the first paper of this series (7). The electrodes used were for the most part purified gold which had been employed in the earlier work.¹ An

¹ The electrodes were placed when not in use in chromic acid solution, and were immersed for several minutes in concentrated sulfuric acid before each experiment. It was found that if this precaution were not taken the electrodes were sluggish and did not agree with one another closely. It was believed that the concentrated acid removed traces of stop-cock lubricant which may have been carried into the cell and become attached to the electrodes, but it is possible that solution of a film of oxide on the surface of the electrodes may have been involved. See Michaelis and Flexner (6).

improvement was made in the method of setting up the agar bridge: instead of filling the tube with agar, sterilizing separately, and fitting it aseptically to the large stopper of the electrode cell, the tube was inserted in the stopper and its outer end connected by rubber tubing provided with a pinch-cock to a funnel containing the KCl agar. After sterilization of the completely assembled apparatus in the autoclave at 10 pounds for 10 minutes, the melted agar was allowed to flow, by opening the pinch-cock, into the bridge tube, while the apparatus was still warm. A drop of agar was allowed to form at the tip of the tube, at which time the pinch-cock was closed. After the agar had solidified the connection to the funnel was removed and replaced by a rubber tube, filled with saturated KCl, which was connected to a special form of calomel half-cell through a three-way stop-cock. During an experiment this cock was kept closed and the rubber tube was shellacked, so that the bridge was a closed system into which oxygen could not readily diffuse. Reliance was placed on careful shellacking of all exposed rubber surfaces for exclusion of oxygen which would otherwise diffuse through the rubber.

The electrode cell and its contents were maintained at 38°C. by means of a small heating coil which fitted closely about the cell. A thermometer was placed in and sterilized with the cell. The flow of electric current through the heating coil was controlled by sliding-contact rheostats; a low-range ammeter in the circuit permitted calibration of the current required to reach and maintain any desired temperature. Although a thermostat was not used the temperature was kept within the limits of $\pm 1.0^{\circ}\text{C}$. variation by adjustment of the rheostats.

The bouillon used was standard meat infusion, adjusted to pH 7.6 and buffered by phosphate which was added to a concentration of $\text{M}/15$. A saturated solution of dextrose was sterilized by heating to 80°C. for 30 minutes; an amount of this sufficient to give a final concentration of 0.5 per cent was added aseptically, in those experiments in which a dextrose medium was used, after the bouillon had been autoclaved. For each experiment 25 cc. of bouillon were autoclaved in a small flask, at 10 pounds for 10 minutes, along with the electrode cell. After cooling to about 38°C. the bouillon was transferred aseptically to the separatory funnel of the cell assembly. While in the funnel the bouillon was inoculated with two loops of an 18 hour broth culture of *B. typhosus* and was then allowed to run into the electrode cell. At intervals during many of the experiments portions of culture were drawn off from the cell by means of the bottom outlet, for the purposes of measurement of pH and of enumeration of bacteria by dilution plate cultures.

The electrical measurements were made by means of the usual potentiometer assembly, and at the same time were followed in many of the experiments by a Leeds and Northrup recording potentiometer. This instrument was specially adapted for this work by the makers; the moving coil has a resistance of 2500 ohms and is delicately balanced. This instrument was used chiefly as a precaution against overlooking changes in potential during the hours when the electrode cell was not under actual observation. The potentials indicated by the automatic

recorder were in general within 5 to 10 millivolts of those determined manually. The numerical values referred to in connection with the description of the experiments represent independent readings taken with the more sensitive hand-operated potentiometer.

Measurement of oxidation-reduction potentials was made first on cultures in bouillon which contained only the small amount of dextrose naturally present. In such cultures the change in pH after 24 hours

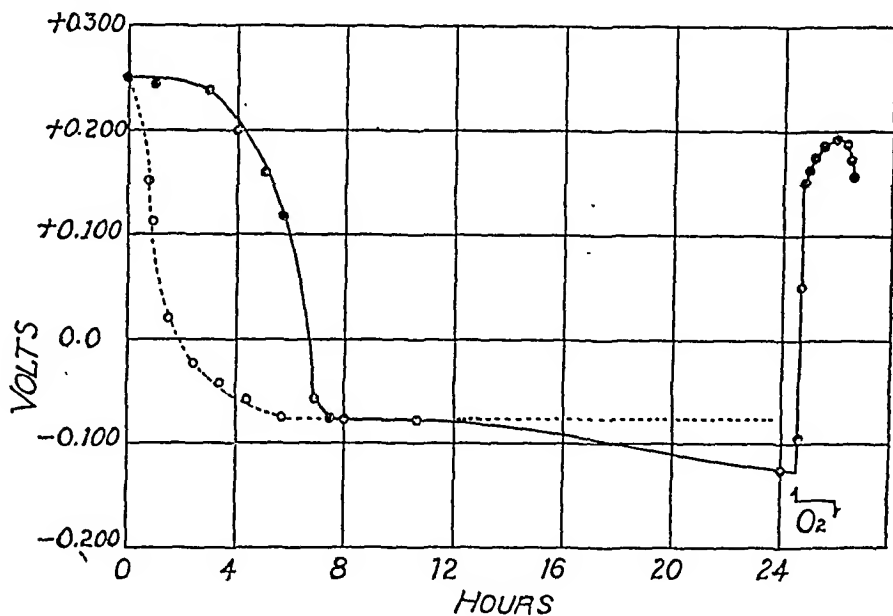


FIG. 1. The potentials of *B. typhosus* in bouillon without added dextrose are given by the solid line. The broken line gives the potentials of the bouillon in the sterile condition when deaerated with nitrogen. The rise in potential of the culture on passage of oxygen and the return to negative values on stopping the flow of oxygen are shown at the right of the figure. The culture throughout incubation had access to atmospheric oxygen. Ordinates represent E_h values at pH 7.6.

growth is small, amounting to less than 0.3 pH; if 0.1 per cent or more of dextrose is present the acid resulting from fermentation of the sugar is sufficient to displace the pH of the medium in spite of the addition of phosphate buffer, and introduces a complication in the interpretation of the potentials observed. When the electrode vessel was half filled with bouillon, and the cocks of the gas tubes entering and leaving the cell were closed, the culture had access to an equal volume of air, and

the conditions were similar to those of the ordinary aerobic culture. The time-potential curves of cultures under these conditions have been obtained in a number of experiments.

The course of the potentials is shown for a typical experiment in Fig. 1. During the first few hours the potentials remain close to the initial values, which range as in sterile bouillon at the beginning of deaeration between $+0.250$ and $+0.150$ volt. The lower initial values have been given by bouillon which had been freshly autoclaved and rapidly cooled before transference to the cell. This is in agreement with the observation of Dubos (8) on the reducing power of sterile media for dyes. A positive drift in potential from a few millivolts to 0.040 volt has usually been observed beginning shortly after measurements were started, and lasting for 2 to 3 hours; it may be related to the equilibration of the freshly autoclaved bouillon with the air. Within 3 to 6 hours after inoculation there has been observed regularly a gradual decline in potential followed shortly by an abrupt drop to -0.040 to -0.070 volt, and a slower fall to -0.080 to -0.090 volt. This level is maintained for several hours and appears to represent an equilibrium value for the culture during its youth. It was not possible, as will be described later, to make frequent samplings of the culture because of the change in potential which these involved, but it is evident from the form of the growth curve in these and in other observations that the culture has passed through its lag period and is in the period of logarithmic increase in numbers of bacilli during the time when this level of potential is maintained. The potentials then undergo a further gradual decline and after 24 hours have been found very close to -0.125 volt. In one experiment in which observations were made over a longer period of incubation this decline continued and at the end of 53 hours the potential reached the value of -0.145 volt.

The time-potential curve of an aerobic culture during the first 24 hours of growth may thus be divided into 4 portions: (1) the potentials are close and slightly positive to the initial values; (2) a decline, in part abrupt, to -0.080 or -0.090 volt; (3) a period in which the potentials are maintained at this level; (4) a gradual decline to more negative values continuing over a period of several days.

It seems highly probable that the presence of oxygen in the culture

is of primary importance in the establishment of the potentials, and the following explanation has been evolved. In the first portion of the time-potential curve, the small numbers of bacteria introduced as inoculum are without effect on the conditions, which are such as to permit the solution of oxygen in the medium. In the second period characterized by the rapid decline in potential, the bacteria have begun to multiply and in their rapid respiration consume the dissolved oxygen of the medium. With logarithmic growth of the bacteria their respiration becomes sufficient to consume the oxygen more rapidly than it can dissolve into the medium, if the surface of the latter is undisturbed, and the actual concentration of oxygen in the system is reduced to a very low value. The potential at the electrode then shows a negative drift just as in sterile bouillon on removal of oxygen by deaeration, and the potential attained is the same as that found in sterile bouillon under anaerobic conditions.

In the earlier work the equilibrium potential of several specimens of sterile bouillon was found to be -0.060 volt. Different specimens vary somewhat in the value of the reduction potential under anaerobic conditions. Separate determinations have been made of the reduction potentials of the bouillon used in the present investigation, in the sterile condition; the values found are somewhat more negative than those previously reported and have fallen between -0.085 and -0.095 volt. Removal of oxygen by the respiration of the bacteria is thus sufficient to explain the attainment of this level of reduction potential in a growing culture.

That removal of oxygen and not the elaboration of reductive products by the bacteria is responsible for reduction potentials of this intensity in bouillon is indicated by the immediate response of the electrode to small amounts of oxygen. If a few bubbles of air or of pure oxygen are allowed to pass through the culture the potentials at once shift in the direction of more positive values, and if aeration is continued there is a return to the neighborhood of the initial values, or about $Eh +0.200$. On stopping the flow of oxygen there is observed again a negative drift which is more rapid than the initial fall that is observed when the culture contains smaller numbers of living bacteria. When small portions of the culture are withdrawn through the bottom outlet of the cell, the disturbance of the culture is apparently sufficient

to increase the rate at which oxygen is dissolved, since the potentials show a positive shift of 0.050 to 0.100 volt. This phenomenon is observed only when oxygen is available for solution by the culture medium, and does not occur when the medium has been deaerated and the space above the bouillon contains nitrogen.

During the third portion of the time-potential curve the potential is maintained at the value characteristic of the sterile bouillon under anaerobic conditions. The duration of this period is uncertain; it appears to be several hours but probably does not exceed 18 hours from the time of inoculation of the culture. The uncertainty is due to the fact that even in bouillon to which dextrose has not been added there is observed an increase in acidity at the end of 24 hours of 0.2 to 0.3 pH, and the true potentials can be obtained only by a correction of the observed potentials for the change in pH. Frequent sampling for the purpose of following the course of the pH was impossible, because of the effect on the potentials which has been described above. For the same reason it was not possible to follow the growth curve closely during this period. It is known, however, that the growth curve of aerobic cultures of *B. typhosus* becomes flattened after about 18 hours of incubation and the rate of dying of the bacteria begins to approach the rate of multiplication. It is highly probable that autolytic processes occur at this time and liberate products of bacterial metabolism into the culture medium. The gradual fall in potential from about -0.085 volt to values between -0.125 and -0.145 volt which takes place following the brief period of maintained low potential may be regarded as a consequence of this autolysis and as evidence of the reductive nature of the substances set free. The data do not permit further characterization of these products; the potentials for which they appear to be responsible are considerably more negative than those observed in the bouillon when maintained in the sterile condition.

It was anticipated that the addition of dextrose to the bouillon would result in the attainment of more negative potentials by the culture than in a medium without added sugar.² This proved not to

² The sterile bouillon used in these experiments gave the same equilibrium potential (-0.085 to -0.095 volt) under anaerobiosis when dextrose was added, in the manner described above, to 0.5 per cent concentration as when dextrose was not added.

be the case. Cultures in 0.5 per cent dextrose bouillon given access to a fixed amount of air yielded time-potential curves very similar to those described above, if correction is made for the shift in pH occasioned by

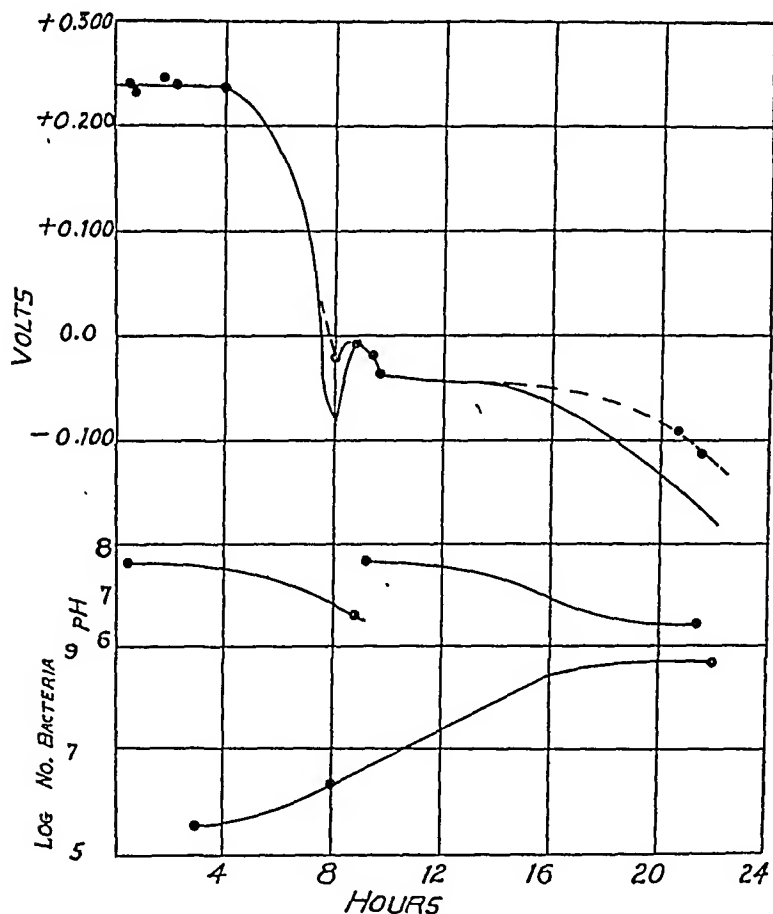


FIG. 2. The upper curve gives the potentials of *B. typhosus* in 0.5 per cent dextrose bouillon. The broken line shows the observed potentials, the solid line the potentials corrected for the change in pH. The positive shift occasioned by sampling of the culture is indicated. The curve of observed potentials represented in part by the broken line was drawn by the recording potentiometer; the experimental points represent values checked by the more sensitive galvanometer. The solid line gives the potentials as corrected for change in pH. The middle curve gives the approximate course of the pH during incubation. The lower curve gives the approximate growth curve. The culture throughout incubation had access to atmospheric oxygen.

acid fermentation. The same effects on the potentials were produced by the admission of air into the culture either deliberately, or incidentally to the disturbance occasioned by sampling, so that the course of reduction potentials and the hydrogen ion concentration could not be followed simultaneously. A typical experiment is shown in Fig. 2. The broken line of the potential curves represents the potentials actually observed; the solid line gives the corrected potentials. The correction of -0.061 volt per unit pH has been applied, on the assumption that no critical points of ionization are uncovered within the range of pH encountered. The potentials show the same rapid drop to more negative values after 6 to 8 hours incubation as in the cultures without added dextrose. The subsequent course of the observed potentials is variable, and in the lack of knowledge regarding the changes in pH, the course of the true or corrected reduction potentials is uncertain. In the experiment recorded in Fig. 2, the pH at the end of 8 hours incubation was 6.6 and the corrected potential was -0.085 volt, which is the value observed at the corresponding period in cultures in which acid fermentation did not occur. At this time the pH of the culture in this experiment was restored to its original value of 7.6 by addition of NaOH. At the end of 22 hours incubation, continued acid production had reduced the pH to 6.7 and the corrected reduction potential was -0.165 volt. This value is 0.040 volt more negative than that observed in cultures without dextrose after the same period of incubation; it suggests that with the increased "turn-over" of bacteria in the presence of dextrose (although the numbers of viable bacteria at any time are no greater than in culture medium without dextrose) a larger amount of reductive substance is liberated from the typhoid bacilli.

The experiments which have been described above indicate that the conditions within an actively growing culture of typhoid bacillus in bouillon are essentially anaerobic, even when the culture has access to atmospheric oxygen. The oxygen which is constantly being absorbed by the culture has an effect on the rate of growth of the bacilli as is shown by experiments which will be reported later, but the actual concentration of oxygen within the medium must be very small. In order to observe the course of the potentials when oxygen is present in high concentration, an experiment was carried out in

which pure oxygen was allowed to bubble through the medium during the period of incubation. The result is shown in Fig. 3, in which the growth curve, and the changes in pH as well as the reduction potentials are recorded. The medium contained 0.5 per cent dextrose. Passage

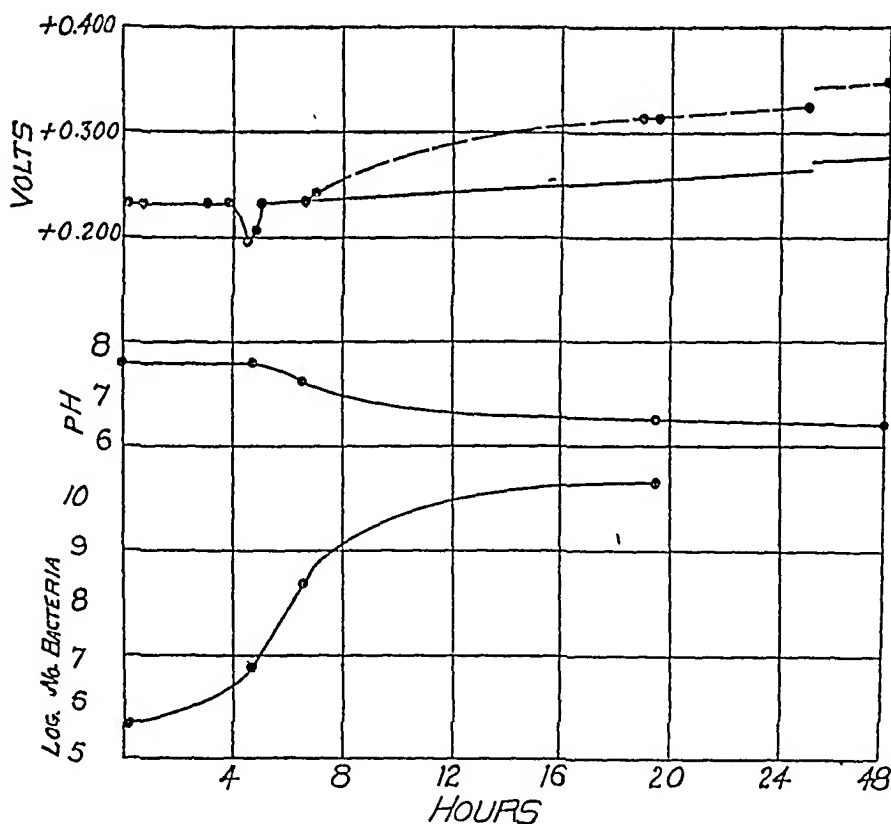


FIG. 3. The upper curve gives the potentials of *B. typhosus* in 0.5 per cent dextrose bouillon. Oxygen was passed through the culture from the time that the early negative drift was observed. The interrupted line represents the observed potentials, the solid line the potentials corrected for change in pH, which is shown in the middle curve. The lower curve represents the growth curve of the bacteria.

of oxygen through the culture was started immediately after the first rapid fall in potential, described above, had begun. The saturation of the medium with oxygen led to an immediate return of the reduction potentials to the initial values. The observed potentials then show a

gradual positive drift; when corrected for the change in pH, a shift to values more positive than those at the beginning of measurement is still evident, although the magnitude of the shift is smaller. The reduction potentials were maintained at a highly positive level throughout 50 hours of incubation. During the first 20 hours of growth the pH shifted from 7.6 to 6.6; the change is less than is observed in cultures which were not aerated. At the same time the numbers of viable bacteria increased enormously. The relations of the oxygen tension to rate of growth and character of metabolism are being made the subject of a separate investigation which will be reported later. In the present connection it is significant that in spite of the enormous numbers of living bacteria, there are not produced reductive substances capable of establishing in the presence of oxygen reduction potentials negative to those of the culture bouillon itself under like conditions. This is not what we should expect if molecular oxygen is inert with respect to reductive substances of bacterial origin. Deaeration at the end of 50 hours incubation, during which saturation of the medium with oxygen had been maintained, led to a rapid drop in reduction potential similar to that observed in cultures which had not been aerated. The equilibrium potential corrected for the shift in pH to 6.4, was -0.080 volt; this is very close to that observed in the sterile bouillon under anaerobic conditions. Growth of the typhoid bacillus in a high concentration of oxygen appears not to lead to the production of substances of greater reducing intensity than those present in the medium before inoculation; in growth under very low oxygen concentration such substances apparently are produced and are liberated into the medium. Further investigation is required to establish the relation between the character of bacterial metabolism, with respect to the processes of respiration and fermentation, and the elaboration of reductive substances.

DISCUSSION AND SUMMARY.

The reducing properties manifested by bacterial cultures must be regarded as only one phase of a series of processes that come within the scope of the concept of oxidation-reduction phenomena. The importance of such processes is suggested in many phases of the metabolism of living cells, as in the accumulation of carbohydrate within

the cell, and the elaboration of other readily oxidized substances such as the hemotoxins studied recently by Neill (9). In these cases the energy of the living cell transforms substances in a state of relatively high oxidation into bodies that are in a condition of low oxidation. Conversely in respiration, as in the familiar oxygen-carbon dioxide exchange, a change in the condition of oxidation of carbon takes place in the opposite direction.

The scope of this concept is therefore very broad and includes other processes than the reduction of dyes and other substances such as nitrates and sulphydryl bodies. Some definite aspect of the general problem must be chosen in order to bring the subject within the range of experiment. In the present series of investigations attention is centered upon those processes of oxidation and reduction which involve electromotively active substances, and are therefore subject to electrical measurement.

In the present paper the oxidation-reduction potentials yielded by cultures of the typhoid bacillus have been observed under the conditions which prevail in the usual aerobic culture in bouillon. Comparison of these potentials with those given by the culture medium in the sterile condition indicates that the living bacteria do not contribute the substances which are responsible for the observed potentials. On the other hand the respiratory consumption of oxygen by the typhoid bacillus appears to render possible the manifestation of characteristic potentials by the culture medium. Such potentials become apparent only when oxygen is removed from the system, and the rôle of the bacteria is the establishment of the anaerobic state.

The potentials of cultures in which the anaerobic condition was maintained from the beginning by artificial means will be reported in a later paper, but it may be said here that the potentials of such cultures confirm the idea of the importance of bacterial respiration in establishing a condition of anaerobiosis in cultures given access to air. The anaerobic cultures develop potentials of the same value as those in which oxygen is available, but attain the level characteristic of the culture medium more rapidly.

This point of view is opposed to that which regards the potentials of bacterial cultures as a manifestation of reductive substances liberated by the bacteria. Since the potentials given by cultures in the

early part of their growth indicate only the level of reduction intensity which the sterile medium may attain, evidence is wanting from this source for the existence of such bacterial products.

In the later stages of growth when the rate of dying of the bacteria approaches the rate of multiplication, the potentials of cultures indicate a level of reduction intensity more negative than that of the culture medium itself. No attempt has been made to work out the exact relations between the growth curve of the bacillus and the reduction potentials since this would involve the detailed investigation proposed by Clark (3). It is certain however that reductive bodies are produced and stored within the cell, as is shown by the reduction of indicators within the living ameba in the experiments of Cohen, Chambers, and Reznikoff (10). The dissolution of cells which must occur in bacterial cultures that have passed the peak of the growth curve would be expected to liberate such reductive substances; if their oxidation is prevented, as in the anaerobic condition which results from respiration of the bacteria, and if in addition the substances are electromotively active, their presence might be indicated by change in the reduction potentials.

It seems possible from consideration of the potentials of cysteine studied by Michaelis and Flexner (6) that among the substances liberated on dissolution of the bacteria are products similar in nature to those already present in the culture medium, so that the more negative potentials may result from an *increase* in the concentration of an electromotively active substance. Other reductive products may be liberated, but nothing can be said at the present time as to their effect on the electrode potentials.

If the substances responsible for reduction potentials more negative than those of the culture medium itself were secreted during growth of the bacteria, rather than liberated by their dissolution, we should expect that their effect on the potentials would be observed during the youth of the culture. The gradual negative drift to values 0.040 to 0.080 volt more negative than those of the sterile bouillon which is observed in cultures after several days incubation indicates that distinct reductive phenomena are associated with the declining portion of the growth curve, in which death of the cells predominates over multiplication.

The failure to attain reduction potentials in cultures saturated with oxygen as negative as those given by cultures which were allowed to develop an anaerobic state may be due to the oxidation of any highly reductive bodies which had been liberated. On the other hand, the enormous numbers of living bacteria in the oxygenated culture suggest that there may be a less rapid "turn-over," and consequently a less extensive dissolution of bacterial cells in such cultures than in those provided with a less abundant supply of oxygen.

The behavior of the typhoid bacillus in the establishment of reduction potentials does not of course permit inference to be drawn as to the behavior of other bacterial species. The observations of Clark (3) show that different species run different courses of potential. It is probable however that any microorganism capable of consuming oxygen as completely as the typhoid bacillus would bring about in bouillon the establishment of potentials as negative as those yielded by the medium itself under anaerobic conditions, unless there is involved an oxidative mechanism which is not evident in the case of *B. typhosus*.

CONCLUSIONS.

1. The reduction potentials of *B. typhosus* in culture in bouillon which is given access to atmospheric oxygen show a negative drift that attains the values found in sterile bouillon when deaerated with nitrogen: E_h -0.085 to -0.095 volt at pH 7.6. The potential reaches this level after 6 to 8 hours incubation, and is maintained at this point for several hours. A slow decline to more negative values is then observed and continues for at least 48 hours, when a potential of -0.145 volt may be attained.

2. The bacteria influence the potentials in the first period of their growth by exhaustion of oxygen from the culture, thus permitting the characteristic potential of the culture medium to become manifest, and do not contribute the substances responsible for the observed potentials. The decline in potential to values more negative than those of the culture medium occurs during the time that the rate of dying of the bacteria approaches and exceeds the rate of multiplication; it is suggested that dissolution of bacteria liberates reductive substances.

3. Cultures in 0.5 per cent dextrose medium show a somewhat more negative potential after 18 hours growth than cultures in medium without dextrose. This may be due to the more rapid "turn-over" of the bacteria and the liberation of larger amounts of reductive material from dissolution of larger numbers of bacteria.

4. The potential of cultures through which oxygen is passed continuously does not show a negative drift at any time. This indicates that reductive substances of bacterial origin in the case at least of the typhoid bacillus do not influence the electrode potentials in the presence of oxygen and confirms the importance of bacterial respiration as the means for the removal of oxygen and the consequent establishment of characteristic reduction potentials in cultures.

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STUDIES ON THE PRECIPITABLE SUBSTANCES OF BACILLI OF THE SALMONELLA GROUP.

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In earlier communications we (1) have described precipitable substances, probably of carbohydrate nature, isolated from *B. typhosus*, *paratyphosus* B, and *enteritidis*, and two antigenic protein preparations obtained from *B. typhosus*. These studies have also been extended to the main serological types of the Salmonella group of organisms.

For a general review of the subject we refer to the communications by P. Bruce White (2), Krumwiede, Cooper, and Provost (3), Tulloch (4), and to the references given in our earlier papers.

We would however like to mention several recent publications. Thus Happold (5) described a precipitable substance obtained from *B. aertrycke* which he considered to be of protein nature and "to be identical with the antigen which stimulates the production of agglutinins to a heat-stable antigenic form of the organism." Ecker and Rimington (6) report obtaining from *B. aertrycke* a carbohydrate containing material possessing toxic properties; while White (7) states that he has extracted a soluble specific substance from *B. aertrycke* similar to the specific soluble substances of Avery and Heidelberger. The precipitable solution obtained was acted upon by the sera of *B. aertrycke* and *paratyphosus* B but not by those of *B. Newport* and *suipestifer*. White's paper contains a full discussion of the serological properties of the rough *B. aertrycke* strains. Casper (8) prepared from *B. paratyphosus* B a carbohydrate containing substance which reacted with sera for *B. paratyphosus* B, *B. typhosus*, and *B. typhi murium*, to a lesser degree with sera for *B. enteritidis* Gärtner, but not with *suipestifer* serum. A report on the carbohydrate and protein fractions of *B. typhosus* was made by Heidelberger, Schwartzman, and Cohn (9).

Preparation of the Crude Precipitable Carbohydrate Substances.—Three methods were employed for the preparation of the crude substances:

1. Extraction by alkaline hypochlorite solution: Bacilli grown on agar for 48 hours were taken up in 0.9 per cent sodium chloride solution, and alkaline hypochlorite solution was added in a quantity sufficient to dissolve the bacteria at about 50°C. The solution was chilled and cold 95 per cent alcohol added until a heavy precipitate was formed carrying down most of the active substance. This

TABLE I.

Precipitation Tests.

Carbohydrates from <i>Bacillus</i>	Antigen dilutions	Immune sera obtained with <i>Bacillus</i>									
		<i>Typhosus</i>		<i>Enteritidis</i>		<i>Paratyphosus B</i>		<i>Derby</i>		<i>Newport</i>	
		+++ ++ + ±	+++ ++ + ±	+++ ++ + ±	+++ ++ + ±	+++ ++ + ±	+++ ++ + ±	+++ ++ + ±	+++ ++ + ±	+++ ++ + ±	+++ ++ + ±
<i>Typhosus</i>	5,000	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++
	50,000	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++
	500,000	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++
<i>Enteritidis</i>	5,000	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++
	50,000	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++
	500,000	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++
<i>Paratyphosus B</i>	5,000	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++
	50,000	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++
	500,000	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++
<i>Derby</i>	5,000	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++
	50,000	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++
	500,000	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++
<i>Newport</i>	5,000	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++
	50,000	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++
	500,000	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++
<i>Hog cholera</i>	5,000	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++
	50,000	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++
	500,000	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++

To 0.1 cc. of the antigen solution 0.05 cc. immune serum and 0.05 cc. physiological salt solution were added. The reactions were read after 2 hours at room temperature (Column 1) and after standing overnight in the ice box (Column 2). The immune sera were obtained by injections of rabbits with heated bacilli, as stated above.

* With other sera of the same type the reactions were negative with the substance of *B. typhosus*.

† With some other sera of the same type marked reactions were obtained with the substance of *B. Newport*.

precipitate was dissolved in water, and, after removal of some insoluble material, was reprecipitated with 95 per cent alcohol. After washing with 95 per cent alcohol, absolute alcohol, and ether, it was dried *in vacuo*.

2. Extraction with saline solution: Bacilli grown on agar for 48 hours were taken up in 0.9 per cent sodium chloride solution, centrifuged,¹ the sediment washed with 95 per cent alcohol, and extracted with boiling 95 per cent, and absolute, alcohol. After filtration on a hot water funnel the bacillary mass was heated in the steam bath two or three times with 0.9 per cent sodium chloride solution for 1 to 2 hours and the extract separated each time by centrifuging. Much of the protein was removed by addition of hydrochloric acid in an amount sufficient to produce maximum precipitation and the fraction was reprecipitated to recover some of the active non-protein substance; the joint mother liquids were then precipitated with alcohol after addition of 1/20 volume of normal sodium hydroxide. The further redissolving, removal of insoluble material, reprecipitation, and drying of the preparation were done as described above.

3. Extraction by autoclaving and subsequent tryptic digestion (*cf.* Goebel and Avery (10)): Agar cultures were washed off and were autoclaved for 20 minutes and digested overnight with purified trypsin solution (Goebel and Avery) at 37°C. The subsequent alcohol precipitation, removal of insoluble material, reprecipitation, and drying of the substance were carried out in the manner given.

Cultures.—Besides our laboratory strains the following cultures obtained through the courtesy of the National Collection of Type Cultures, London,² were used: *B. paratyphosus* B, Tidy No. 14; type Stanley, No. 92; type Reading, No. 72; type Derby, No. 1729; type Newport, No. 129; type Hog cholera, No. 356; *B. enteritidis*, Gartner, No. 127; *B. abortus-equinus*, No. 766; and *B. aertrycke*.³ They were plated on agar and colonies showing homogeneous growth in broth were selected.

Immune Sera.—Unless otherwise stated, immune sera were prepared by weekly injections of rabbits with about 1/10 to 1/20 of 24 hour agar slant cultures heated to 62–65°C. for 40 minutes. Immune sera prepared with various strains of the same type, e.g. *B. typhosus*, showed considerable variations in their capacity to precipitate the carbohydrate preparations.

Precipitin Reactions of the Carbohydrates and Their Parallelism to the "Small Flaking" Agglutination.—Precipitin reactions with the purified specific substances (see page 737) are presented in Table I. The crude substances had a lower titer but showed in cross-tests almost the

¹ In a control experiment the bacillary mass was washed several times with saline solution.

² We are indebted to Dr. R. St. John-Brooks, Curator of the National Collection of Type Cultures, London, for supplying us with the cultures mentioned.

³ The numbers correspond to those given in the catalogue of the collection.

same range of specificity as purified preparations, no matter which of the methods described was employed.

It is seen that the strains can be divided roughly into three groups within which the strongest cross-reactions occur. The first group consists of *B. typhosus* and *B. enteritidis*, the second of *B. paratyphosus*

TABLE II.*

<i>Typhosus</i>	<i>Enteritidis</i>	<i>Paratyphosus</i> B	<i>Derby, Reading, abortivo-equinus</i>	<i>Newport</i>	<i>Hog cholera</i>
III, (X), 8	III, 8	I, II, 7, 8	II, 7, 8	IV, VI, 7	V, VI

* Roman numerals are used by White to designate salient components, Arabic numerals for those of minor development.

TABLE III.

Absorption Tests with Immune Serum for B. typhosus.

Dilution of the carbohydrates	Serum, unabsorbed		Serum absorbed with alcohol-treated <i>B. enteritidis</i>	
	Precipitation tests with the carbohydrate of <i>B. typhosus</i>			
5,000	++	+++	0	0
50,000	++	++	0	0
500,000	f. tr.	tr.	0	0
Dilution of the immune serum	Agglutination tests with <i>B. typhosus</i> (suspension preserved with chloroform)			
300	+++	+++	+++	+++
900	+++	+++	++	++
2,700	++	++	+±	+±
8,100	+	+	±	tr.
24,300	0	tr.	0	0

The reactions were read after 1½ hours at room temperature (Column 1) and after standing overnight in the ice box (Column 2).

B. and *B. Derby*, and the third of *B. Newport* and *B. hog cholera*. Additional tests with crude preparations, not included in Table I, showed that the substances and sera of *B. Stanley*, *B. Reading*, and *B. abortivo-equinus* reacted like those of *B. paratyphosus* B.

A comparison of these results with the agglutinin reactions of the "stable agglutinogens" suggested itself. The distribution of the

agglutinable factors underlying the "small flaking" agglutination is summarized, according to White, in the scheme shown in Table II.

On comparing this scheme with the precipitin reactions an approximate correspondence between the stable agglutinogens and the precipitable carbohydrates appears (see White).

This relation is also illustrated by the following observations (Table III): An immune serum for *B. typhosus* was absorbed with alcohol-treated *B. enteritidis* and tested for precipitins and agglutinins to *B. typhosus*. Since the "stable" alcohol-resistant agglutinogens of *B.*

TABLE IV.
Absorption Experiments.

Dilution of the precipitable substance	Precipitable substance	Immune serum for <i>B. enteritidis</i>		Precipitable substance	Immune serum for <i>B. Newport</i>		Precipitable substance	Immune serum for <i>B. hog cholera</i>	
		Unabsorbed	Absorbed with <i>B. paratyphosus</i> B		Unabsorbed	Absorbed with <i>B. hog cholera</i>		Unabsorbed	Absorbed with <i>B. Newport</i>
2,000	<i>Enteritidis</i>	+++	+++	<i>Newport</i>	+++	+++	<i>Hog cholera</i>	+++	+++
20,000		+++	+++		+++	+++		+++	+++
200,000		±	±		+	+		+++	+++
2,000	<i>Paratyphosus</i> B	+++	0	<i>Hog cholera</i>	++	0	<i>Newport</i>	+	0
20,000		+++	0		+++	0		±	0
200,000		±	0		+++	0		tr.	0

The immune sera were absorbed with bacillary suspensions kept in alcohol and washed once with saline solution.

typhosus and *B. enteritidis* are supposed to be very similar it was to be expected that by the exhaustion with alcohol-treated *B. enteritidis* the precipitins would be removed but not the "large flaking" agglutinins. The result of the experiment bore out this assumption.

In contrast with the absence of precipitating action of fluids with large flaking agglutinins of high titer (*cf.* Heidelberger, Schwartzman, and Cohn) are instances in which immune sera with a relatively low agglutinin content, mainly of the small flaking type, precipitated intensely the carbohydrate solutions. Such sera were prepared by the method of Douglas and Fleming (1).

Some discrepancies will be noted such as the absence of precipitin reactions of certain *B. paratyphosus* B sera on the substances of *B. Newport* (factor 7) and *B. typhosus* (factor 8), and the lack of precipitating capacity of most of the hog cholera sera for the substance derived from the strain *Newport* (factor VI). Since in these combinations some of the sera give positive reactions one may assume that the precipitable substances do not lack the properties in question. An explanation may perhaps be seen in the observation that the agglutinin reactions with alcohol-treated bacilli were comparatively weak in

TABLE V.

Precipitable substance	Dilution	Immune serum for <i>paratyphosus</i> B				
		Unabsorbed, diluted			Absorbed with <i>B. Reading</i> , diluted	
		1:2	1:8	1:32	1:2	1:8
<i>Paratyphosus</i> B, not treated with alkali	5,000	+++	+++	+	++++	+++
	50,000	+±	++	++	++	++
	500,000	±	±	tr.	±	—
<i>Paratyphosus</i> B, treated with alkali	5,000	++	+	tr.	0	—
	50,000	+	+	0	0	—
	500,000	f. tr.	0	0	0	—
<i>Reading</i> (treated with alkali)	1,000	+++	++	tr.	0	—
	5,000	++	+±	f. tr.	0	—
	25,000	+	+	f. tr.	0	—

those instances in which precipitation was lacking. However, a thorough study of this question was not made.

In order to determine whether the multiplicity of precipitins corresponds to that of the agglutinins, absorption experiments were made in several cases (Table IV).

On the whole the tests were in agreement with the idea of a multiplicity of precipitins, corresponding to that of agglutinins. A striking exception was the following:

Immune sera for *B. paratyphosus* B or *B. Stanley*, when absorbed with *B. Reading* or *B. abortivo-equinus* did not precipitate the substances prepared from the homologous organisms, although they agglutinated the alcohol-treated bacillary suspensions. Search for

the missing precipitable property (attributable to factor I) revealed that it was present in the crude saline extracts, but disappeared after treatment with alkali. Substances exhibiting the property were prepared as follows:

Saline extracts obtained from alcohol-extracted bacilli, as described above, were precipitated with alcohol (without the addition of alkali), after previously removing the substances precipitable by dilute acid. The precipitate was dissolved in a small volume of water, some insoluble material discarded, and 50 per cent trichloroacetic acid added to cause optimal precipitation. The unneutralized mother liquid containing most of the active substance was then precipitated with an excess of 95 per cent alcohol and dried after washing several times with 95 per cent alcohol, absolute alcohol, and ether.

Table V indicates that immune sera for *B. paratyphosus* B (or *B. Stanley*) contain a fraction of antibodies directed towards the alkali-labile property I. After absorption with *B. Reading* the immune serum for *B. paratyphosus* B still precipitates the substances derived from this bacillus but no longer acts on the substance from *B. paratyphosus* B after treatment with alkali. That this effect is not due to a diminution of one single antibody was shown by tests with diluted immune serum.

The reactivity of the substance was not altered by peptic and tryptic digestion at a pH not in itself injurious. It was not destroyed by treatment for 1 hour at room temperature, with normal hydrochloric acid, but it did not withstand under the same conditions the action of 0.01 normal sodium hydroxide (Table VI).

A preliminary investigation was made of the carbohydrates of "rough" strains of *B. paratyphosus* B and *B. aertrycke*. According to several authors such strains may contain special antigenic components. In view of these statements we prepared precipitable substances from "rough" strains by the method of dissolving the bacilli in alkaline hypochlorite solution (see page 727). The substance prepared from *B. aertrycke* "R" and purified as described below proved to contain large amounts of carbohydrates not further investigated. In precipitation tests (Table VII) the "R" substances reacted specifically with the immune serum to the "R" strains whereas the corresponding "S" preparations reacted also to "R" immune sera. It is uncertain whether the interaction can be explained by the presence of "R" forms in the "S" strains.

Attempts to Fractionate the Precipitable Substances by Means of Precipitins.—The resistance of the specific precipitable substances to the action of alkali seemed to offer a way of ascertaining whether the various serological properties of these substances are due to different

TABLE VI.

Precipitin Tests Showing the Effect of Acid, Alkali, Peptic, and Tryptic Digestion on Factor I of B. paratyphosus B.

Dilution of the precipitable substance	Control I	0.01 N NaOH	N HCl	Peptic digestion	Control II	Tryptic digestion I	Tryptic digestion II	Control III
5,000	++++	0	+++	+++	+++	+++	0	0
100,000	+	0	±	+	+±	+	0	0

To 0.1 cc. of the precipitable substance, 0.1 cc. of an antibody solution for factor I (immune serum to *B. paratyphosus* B absorbed with suspensions of *B. Reading*) was added.

Control I: untreated precipitable substance.

Control II: 0.5 cc. of a 1 per cent solution of the substance, 0.05 cc. N HCl, 0.45 cc. water; kept at 37° for 24 hours.

Control III: 0.5 cc. of a 1 per cent solution of the substance, 0.2 cc. of a 1 per cent solution of Na_2CO_3 , 0.8 cc. water; kept at 37° for 24 hours.

Peptic digestion: 0.5 cc. of a 1 per cent solution of the substance, 0.05 cc. N HCl, 0.1 cc. of a 0.2 per cent pepsin solution, 0.35 cc. water; kept at 37° for 24 hours.

Tryptic digestion I: 0.5 cc. of a 1 per cent solution of the substance, 0.5 cc. of a 1 per cent trypsin solution, 0.05 cc. 1 per cent Na_2CO_3 , 0.45 cc. water; kept at 37° for 24 hours.

Tryptic digestion II: 0.5 cc. of a 1 per cent solution of the substance, 0.5 cc. of a 1 per cent trypsin solution, 0.2 cc. 1 per cent Na_2CO_3 , 0.3 cc. water; kept at 37° for 24 hours.

For the treatment with acid and alkali a 2 per cent solution of the substance was mixed with an equal volume of 0.02 N NaOH or 2 N HCl respectively and the tests kept for 1 hour at room temperature.

separable portions. Accordingly attempts were made to fractionate the precipitable substances in a manner analogous to the usual absorption experiments with immune sera.

By suitable exhaustion with heterologous bacilli fractions of immune sera were prepared acting only on part of the supposed precipitable factors. The precipitates produced were washed twice with saline

solution and boiled for a few seconds with a small quantity of 0.1 normal sodium hydroxide solution, the solutions obtained being neutralized and centrifuged.

When such solutions were tested with various antibodies they behaved like the original precipitable substance and not like qualitatively different fractions thereof.⁴ With the procedure described no difficulty was encountered in separating precipitable substances of two different strains of bacilli after their solution had been mixed. It was

TABLE VII.
Precipitation Test.

Immune serum of <i>Bacillus</i>		<i>Aertrycke</i> "S"		<i>Aertrycke</i> "R"		<i>Paratyphosus</i> B "S"		<i>Paratyphosus</i> B "R"	
Precipitable substance	Dilutions								
<i>Aertrycke</i> "S"	2,000	+++	+++	±	±±	±	±	±	+++
	20,000	++	++	±	++	+	++	tr.	+
	100,000	±	+	f. tr.	+	±	±±	0	tr.
	500,000	0	0	0	0	0	0	0	0
<i>Aertrycke</i> "R"	2,000	0	0	±	±±	0	0	++	±±±
	20,000	0	0	0	0	0	0	tr.	+
<i>Paratyphosus</i> B "S"	2,000	+++	+++	++	±±±	+	++	++	++
	20,000	++	±±±	+	++	±	++	tr.	±±
<i>Paratyphosus</i> B "R"	2,000	0	0	++	+++	0	0	+++	+++
	20,000	0	0	++	+++	0	0	++	±±±

Reactions read after 2 hours (Column 1) and after standing overnight (Column 2).

thought however that in these control experiments the mixture of the substances might not have been as intimate as that of the hypothetical fractions in the precipitable carbohydrates derived from one organism. We attempted therefore to separate by the same method precipitable substances after their solutions had been mixed, boiled, precipitated with alcohol, and dried in the usual manner.

⁴ The results were the same in experiments aiming at the separation of Factors I and II of the precipitable substances of *B. paratyphosus* B. In this instance, instead of alkali, dilute acid was used for decomposing the precipitate.

TABLE VIII.

Precipitable substance	Dilution	Immune sera for:							
		<i>B. paratyphosus</i> B absorbed with <i>B. enteritidis</i> (II-7)		<i>B. enteritidis</i> (8)		<i>B. hog cholera</i> absorbed with <i>B. Newport</i> (V)		<i>B. Newport</i> (VI)	
Mixture of the carbohydrates of <i>B. paratyphosus</i> B and <i>B. hog cholera</i>	1,000	+±	++++	+	++++	++	++++	++	++++
	10,000	++	++++	±	++±	++±	++±	++±	++++
	100,000	tr.	+	f. tr.	±	f. tr.	tr.	f. tr.	tr.
Factor II, 7	1,000	+±	++±	+	++±	0	0	0	0
	10,000	±	++±	tr.	±	0	0	0	0
	100,000	0	0	0	0	0	0	0	0
Factor 8	1,000	+±	+++	+	+++	0	0	0	0
	10,000	tr.	tr.	0	f. tr.	0	0	0	0
	100,000	0	0	0	0	0	0	0	0
Factor V	1,000	0	tr.	0	0	+++	+++	+++	+++
	10,000	0	0	0	0	±	+	±	±
	100,000	0	0	0	0	0	tr.	0	tr.
Factor VI	1,000	0	f. tr.	0	0	++	++±	++±	++±
	10,000	0	0	0	0	±	+	±	+
	100,000	0	0	0	0	0	0	0	0

To a 0.1 per cent solution of the mixture of *B. paratyphosus* B and *B. hog cholera* carbohydrates an equal volume of the immune sera (diluted 1:2) was added. After keeping the tubes for 2 hours at room temperature and overnight in the ice box, the precipitate formed was dissolved by sodium hydroxide, as described, and brought with saline solution to half of the original volume. (This dilution was arbitrarily designated 1:1000.)

Factor II, 7: precipitate formed by *paratyphosus* B immune serum absorbed with *B. enteritidis*.

Factor 8: precipitate formed by immune serum of *B. enteritidis*.

Factor V: precipitate formed by hog cholera serum absorbed with *B. Newport*.

Factor VI: precipitate formed by immune serum of *B. Newport*.

In these tests a considerable amount of carbohydrate was carried down non-specifically by the heterologous serum. The possibility that this effect was caused by imperfect solution of the mixture of precipitable substances led to the following modification of the experiment. The dried mixture obtained as before was dissolved in 0.1 normal sodium hydroxide, boiled for 1 hour, and neutralized. In this way again by specific precipitation an almost complete separation could be brought about.

In the following experiment this method was applied to the separation of fractions of single precipitable substances (Table VIII).

The experiment shows that while the two substances mixed together could be separated, apparently no fractionation of either of them was accomplished.

An analogous experiment was carried out with the substance of *B. Newport* and with the sera to *B. suipestifer* (factor VI), *B. paratyphosus* B (factor 7), and *B. Newport*, after exhaustion with *B. suipestifer* and *B. paratyphosus* B (factor IV). Comparing factors IV and VI the results resembled those just reported; the substance however precipitated by *paratyphosus* B serum gave a considerably stronger reaction with this serum than with the hog cholera serum. Conversely the solution obtained from the precipitate caused by the hog cholera serum reacted, like the original substance, more intensely with this serum than with *B. paratyphosus* B serum.

Chemical Data on the Precipitable Substances.—Several of the substances described were purified to a certain extent by a procedure very similar to that used for pneumococcus polysaccharides by Avery, Heidelberger, and Goebel (11).

The crude preparations were dissolved in water, some insoluble matter removed, and, after addition of normal sodium hydroxide to a concentration of about N/20, the active substance was precipitated with alcohol. This precipitation was repeated 4 to 6 times. Usually sodium acetate was added to aid flocculation. Then the solution was acidified with hydrochloric acid and precipitated with acidulated alcohol. When possible the precipitate was made in two steps, the first precipitate, containing most of the proteins and much carbohydrate, was removed by centrifugalizing and the supernatant fluid poured into an excess of acidulated alcohol. From the first pre-

cipitate, rich in proteins, part of the carbohydrate could be recovered by repeating the procedure.

The carbohydrate substance was reprecipitated in acid solution and dried after washing with alcohol and ether. The solutions were kept at low temperature while in acid solution.

These fractions were soluble in water, strongly reduced Fehling's solution after hydrolysis, and gave faint or negative protein reactions, particularly no precipitate with tannic acid or uranyl nitrate. The purified substance of *B. typhosus*, unlike the crude preparation, was not precipitated by barium hydroxide.

On analysis, the figures for the N content of the substances prepared from *B. typhosus*, *B. enteritidis*, *B. paratyphosus* B, *B. Derby*, *B. Newport*, and *B. hog cholera*, varied from 0.5 per cent to 1.4 per cent. The values for $[\alpha]^D$ were as follows: *B. typhosus*, +103; *B. enteritidis*, +95; *B. paratyphosus* B, +94; *B. Derby*, +76; *B. Newport*, +75; *B. hog cholera*, +48. For the sugar analysis the substances were hydrolyzed by heating 1 per cent solutions with an equal volume of normal hydrochloric acid in the steam bath for 5 hours, and the sugar content was determined by reduction of Fehling's solution. It was not established whether the time chosen was sufficient for complete hydrolysis. The values obtained were between 63 per cent and 74 per cent (calculated as glucose) for the six strains mentioned. During hydrolysis with acid some insoluble material, partly soluble in alcohol and of acid character, separated from the solutions as in the case of the specific substance from *V. cholerae* (12). No conclusion can be reached as yet whether these products form a part of the precipitable substances or are impurities.

Since it seemed possible that the precipitable substances contained carbohydrates derived from the agar used for cultivating the bacteria, two preparations were made from gelatin cultures of *B. typhosus* and *B. paratyphosus* grown in Blake bottles. They gave the following figures: *B. typhosus*, reducing sugar 69.5 per cent, $[\alpha]^D$ + 98°; *B. paratyphosus*, reducing sugar 67.3 per cent, $[\alpha]^D$ + 99°. It cannot be claimed that the substances were obtained in a state of purity and therefore all of the figures given are to be considered as preliminary.

On testing the action of alkali and acid it was found that the precipitable substances were remarkably resistant to alkali but readily destroyed by acids.

TABLE IX.

TABLE IX.

Carbohydrates	Dilution	Heating with N HCl for	15 min.	Heating with N NaOH for	2 hrs.	Control untreated	
From <i>Bacillus</i>		3 min.	f. tr.	½ hr.	0		
		0	0	0	0	+	+
<i>Proteus</i> OX ₁₉	10,000	0	0	0	0	+	+
	100,000	0	0	0	0	+	+
<i>V. cholerae</i>	10,000	±	+	+	+	+	+
	100,000	0	+	±	0	+	+
<i>Pneumococcus</i> Type III	10,000	+	+	+	+	+	+
	100,000	+	±	+	±	+	+
<i>B. typhosus</i>	10,000	0	0	—	+	+	+
	100,000	0	0	—	±	+	±
<i>B. enteritidis</i>	10,000	0	0	—	+	+	+
	100,000	0	0	—	+	+	+
<i>B. paratyphosus</i> B	10,000	0	f. tr.	—	+	+	+
	100,000	0	0	—	+	+	+
<i>B. Derby</i>	10,000	0	0	—	+	+	+
	100,000	0	0	—	+	+	+
<i>B. Newport</i>	10,000	0	0	—	+	+	+
	100,000	0	0	—	+	+	+
<i>B. hog cholera</i>	10,000	±	+	—	+	+	+
	100,000	0	+	—	±	+	±

The extraction with boiling 75 per cent alcohol (1,10).

The substances of *Proteus* OX₁₉ and cholera were prepared by extraction with boiling 75 per cent alcohol (1,10). The specific polysaccharide of *Pneumococcus* III was obtained through the courtesy of Dr. Avery.

The first reading of the precipitin tests was made after 2 hours at room temperature, the second reading after the tests were kept overnight in the ice box.

To a 2 per cent solution of the various preparations an equal volume of 2 normal sodium hydroxide or 2 normal hydrochloric acid was added and the solutions were kept in boiling water.

It is seen from the experiment (Table IX) that the preparations of the *typhosus-paratyphosus* group were similar as regards their resistance to acid and alkali, with the exception of the hog cholera substance which was somewhat more resistant to acid than the other preparations. The substances from the other organisms tested, behaved differently. Thus the preparation obtained from *Proteus* O_{x19} was destroyed by alkali as well as by acid, under the conditions of the experiment; the Pneumococcus III preparation was resistant to both, whereas the substance derived from *V. cholerae* was less resistant to alkali than the Salmonella carbohydrates. The differences found would seem to be significant even if one takes in account the variation in the method of preparation.

Observations on the Precipitable Proteins of B. typhosus.—A precipitable protein was prepared in a similar manner as the preparation P₂ *typhosus* described previously (1). The precipitation with alcohol of the extracted substance was omitted but after removal of the suspended bacilli by centrifugalizing, the saline extract was precipitated with dilute hydrochloric acid, redissolved by addition of a small quantity of alkali, filtered through a Berkefeld filter, and reprecipitated with acid. The precipitate was dried after washing with alcohol and ether.

This substance when injected into rabbits induced the formation of "large" and "small" flaking agglutinins aside from precipitins. Although this would seem to point to a relation between the substance P and "large flaking" agglutinogens there are observations which do not agree with this assumption. In the first place the titer of the large flaking agglutinins was relatively low in comparison with sera obtained with bacillary suspensions, and on prolonged immunization the increase of precipitins was not accompanied by a corresponding rise in the agglutinin titer. Furthermore in an absorption experiment the large flaking agglutinins of the sera for P₂ were apparently absorbed to a greater extent by typhoid bacilli treated with alcohol than the large flaking agglutinins of common typhoid sera.

It is as yet difficult to interpret these observations. They may possibly be ascribed to the presence in the P₂ preparations of a special substance responsible for the production of flagellar agglutinins, or to some flagellar material perhaps in an altered state which passed the filter candles. In this respect attention may be called to the observation that bacilli treated with alcohol are no longer agglutinable by large

flaking sera although they give rise to the formation of large flaking agglutinins (13).

The toxic action of the preparation P_2 , mentioned previously, showed considerable variation. One preparation was toxic for rabbits in a dose of 0.5 mg. given intravenously. On repeated injections the animals tolerated doses up to about 20 mg. These animals as well as those immunized with digested bacilli (14) exhibited a typical Arthus phenomenon on intradermal injections of about 1 mg. of P_2 or the carbohydrate respectively. In cross-tests the reactions with the homologous substances were more pronounced. (Cf. the experiments on anaphylaxis by Tomcsik (15) and Avery and Tillett (16).) These tests were made on a small number of animals and therefore should be considered as preliminary.

DISCUSSION.

The carbohydrate-containing preparations isolated from the main serological types of the Salmonella group gave on analysis figures for nitrogen of 0.5 to 1.4 per cent, but they showed only weak or negative reactions for proteins. One may assume that their serological activity is due to specific carbohydrates, for during purification there was a diminution of the nitrogen content and of the protein reactions along with an increase in the amount of sugar liberated by hydrolysis and an increase of the serological activity. The specific reactivity of the preparations to immune sera remained almost unimpaired after heating to about 100° with normal alkali for 2 hours but was quickly destroyed by boiling with normal hydrochloric acid; parallel with the disappearance of the serological activity reducing sugar and some insoluble material were set free. Aside from these observations the assumption that the substances are carbohydrates rests on the analogy of the results with those of Avery, Heidelberger, and Goebel (cf. 11).

The present studies support the view that the specific carbohydrates form an essential part of the "stable" agglutinogens of the bacilli (White (7)). We failed to establish a relationship to the phenomenon of large flaking agglutination.

The similarity of agglutination and precipitation is also shown by absorption experiments, which demonstrate that from one immune serum precipitins can be separated which correspond to agglutinin fractions (cf. Krumwiede (17)).

The explanation of these phenomena so far as the antigens are concerned is still an open one. The fact that certain antibodies are removed from an immune serum through successive absorptions and others are left behind gives evidence for a multiplicity of antibodies; but when the conclusion is drawn from the reaction of certain antibody fractions upon several antigens, that all of the positively reacting antigens contain a definite common substance or one clearly defined chemical group, it remains hypothetical so long as the assumed different elements have not been separated or established as individual structures by chemical methods (see 18). It could also be assumed that the phenomena are at least in part brought about by the action of one antibody on several antigens whose specific groups are similar but not identical (see 19, 4).

In order to examine the question raised, an attempt was made by specific precipitation to separate the carbohydrates into their hypothetical units. On the whole these experiments did not lead to obtaining fractions with different properties; and therefore the results did not support the idea of the existence of separable units in the single antigens. Indeed it would be desirable also to apply chemical methods for the purpose of fractionation of the specific carbohydrates.

A noteworthy difference between the observations reported and the results obtained with the carbohydrates of pneumococci by Heidelberger, Avery, and Goebel, is the following: The carbohydrates of the three fixed types of pneumococci exhibit very marked chemical differences in correspondence with their serological diversity. Such conspicuous chemical differences have not been found among the carbohydrate preparations from the Salmonella group, which, although serologically different, showed no very striking variation in sugar content and optical rotation, with perhaps one exception (*B. hog cholera*).

SUMMARY.

Specific precipitable substances rich in carbohydrates, containing very little protein and small amounts of a material apparently of fatty nature, have been prepared from the main serological types of the typhoid-paratyphoid groups. The preparations in their present

state of purity do not exhibit very pronounced chemical differences in spite of serological dissimilarity. In this respect the results differ from those observed with the polysaccharides of pneumococci.

The specificity of the precipitin reactions of these substances parallels in a general way the so called small flaking agglutination.

Attempts to separate different fractions from the active substance serologically by means of precipitation with antibody solutions were on the whole unsuccessful.

The differences in resistance to the action of acid and alkali were found to be characteristic for various specific carbohydrates.

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THE PRODUCTION OF PARTIAL LIVER INSUFFICIENCY IN RABBITS.

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Reasons enough for the existence of the liver are known. A host of functions have been ascribed to the organ already. Nevertheless the clinical condition of liver insufficiency is still a riddle. For the better understanding of it one must distinguish between functions which are essentially hepatic and those which can be carried out somewhere else in the body when the liver fails.

The recent work on dogs by Mann (1-9) and his colleagues has greatly increased our knowledge of liver functions. But much remains to be learned, and the method of these authors is laborious. For this reason we venture to submit a simple procedure for inducing extreme liver insufficiency in rabbits.

To approach the problem of liver insufficiency in animals three general types of procedure or their combination are available; total extirpation, partial ablation, and derangement of the organ by tying the bile duct or by chemical injury. The method of total extirpation, though it has served most excellently (1-9), still leaves much to be desired. Death follows the operation so rapidly that scant time remains for study of its onset. The use of drugs to bring about liver derangement is questionable for one must assume that the substance employed acts only on the liver parenchyma and not elsewhere as well. The evidence for and against these methods has been reviewed by Marshall and Rowntree (10) and by Mann (9).

The employment of partial ablation has also been open to objection. Investigators are agreed (9, 11, 12) that the liver possesses a "factor of safety" so great that it is well-nigh impossible to remove enough parenchyma to produce insufficiency without fatally obstructing the portal flow. Workers partially ablating the liver in dogs and rats

have found this difficulty insurmountable (9, 11, 13). In the rabbit our efforts have led to success. One can remove slightly more than 90 per cent of the animal's liver without untoward obstruction to the portal blood flow, with result in an extreme degree of hepatic insufficiency. The animals unless treated succumb in 12 to 18 hours, with convulsions and low blood sugar. If given glucose they live for varying periods up to 5 days, eventually dying with symptoms like those exhibited by dogs deprived of the entire liver (2, 9). For a study of the problems of liver insufficiency the method presents many advantages. Not the least of these is the ease of the ablation. It can be accomplished in less than 10 minutes, by a single operator, unassisted.

Anatomical Peculiarities of the Rabbit's Liver.

The arrangement of the rabbit liver in well-nigh separate lobes permits the removal of much of the hepatic substance by simple ligations and excision. Three large cephalad lobes, comprising what has been termed the "main liver" (14-16), constitute about 70 per cent of the organ. The remainder, almost separate from these, the "posterior lobe mass" (14, 15), is situated further from the head and to the right of the portal vein. Unlike the lobes of the "main liver" the "lobe mass" is intimately attached to the vena cava and cannot be wholly ablated without injury to this vessel. But a deep cleft exists on the cephalad surface of the mass and here a ligature may be thrown about the lobe and tied in such a way that it lies close to the vena cava but yet not close enough to impede the flow of blood. The half of the lobe beyond this ligature can now be removed, leaving a neat small stump. The small caudate lobe on the other side of the cava possesses a flattened, tail-like portion springing from a narrow base which can be readily severed after ligation. There remains about half the substance of the lobe, that portion which extends along the sheath of the portal vein between it and the spinal column.

In our attempts to induce liver insufficiency in the rabbit we employed three types of sub-total hepatectomy. Ablation of the "main liver" was the first. To this operation there was next added excision of the removable part of the caudate lobe, leaving the stump and the whole "posterior lobe mass" intact. Finally removal of the "main

liver" and half of the "posterior lobe mass" was practiced, leaving only the stump of this latter and the small caudate lobe intact. This last operation alone resulted in true liver insufficiency.

Estimation of the Relative Amounts of Liver Tissue Removed by the Three Types of Sub-Total Hepatectomy in the Rabbit.

In twenty-five normal rabbits we have separated the portions of the liver, discussed above, weighing the "main liver," the excisable portion of the "posterior lobe mass," its remaining stump, the removable bit of the caudate lobe, and its stump. Table I shows, in terms of percentage of total liver, the relative amounts of the tissue remaining and removed by each of the three procedures outlined above. Further, the table gives some idea of the relative variability in individual instances. Simple ablation of the "main liver" (see Column I of the table) withdraws on the average about 73.6 per cent of the liver tissue, a figure well in accord with the findings previously published by Rous and Larimore, 72.3 per cent (15), and by Ponfick, 74.7 per cent (16). As will be seen below, this procedure does not induce apparent liver insufficiency. "Main liver" removal plus partial ablation of the caudate lobe offers no great additional advantage over the former method, for the caudate lobe adds but little to the percentage of tissue removed (Column II). Ablation of the "main liver" and half the "posterior lobe mass" is the method of election (see Column III of the table). By this procedure an average of slightly over 90 per cent of the liver tissue is removed, the remaining stump of the "posterior lobe mass" and the intact caudate lobe constituting but 9.6 per cent of the original total.

Method.

Rabbits weighing 1500 to 4500 gm., previously kept on a mixed diet, were fasted but allowed water for periods of 24 to 48 hours. They were then operated upon under ether anesthesia. The three large cephalad lobes of the liver, the "main liver," were removed together with that portion of the "posterior lobe mass" which lies to the right of the vena cava.

To accomplish this, the "posterior lobe mass" of the liver was exposed and the ligament attaching its lower medial pole to the sheath of the vena cava severed. A heavy silk ligature was thrown around the lobe, placed in the deep incisura on its cephalad surface, and tied, care being taken to avoid all puckering of the vena cava or inclusion of its sheath in the knot. The liver portion to the right of the

TABLE I.

The Relative Amounts of Liver Removed by the Three Methods of Sub-Total Hepatectomy Described in the Text.

		I				II		III	
	No.	Body wt.	Liver percent- age of body wt.	"Main liver" out		Posterior lobe in		Caudate lobe in	
				Per cent of total liver		Per cent of total liver		Per cent of total liver	
				Out	In	Out	In	Out	In
Freshly fed		gm.							
	1	2570	2.32	76.8	23.2	78.6	21.4	93.4	6.6
	2	1960	2.70	72.7	27.3	76.1	23.9	90.8	9.2
	3	2150	2.21	68.1	31.9	72.2	27.8	90.7	9.3
	4	2957	2.41	76.5	23.5	81.2	18.8	90.2	9.8
	5	2250	2.88	81.2	18.8	83.9	16.1	89.4	10.6
	6	2010	2.22	76.2	23.8	81.0	19.0	89.1	10.9
	1a	4275	2.48	73.7	26.3	77.2	22.8	91.9	8.1
	1b	2375	2.36	80.1	19.9	84.2	15.8	91.2	8.8
Average.....		2568	2.45	75.7	24.3	79.3	20.7	90.8	9.2
Fasted 24 hrs.	1	1980	4.38	73.1	26.9	76.8	23.2	91.4	8.6
	2	2310	4.61	75.3	24.7	80.2	19.8	90.6	9.4
	3	2621	4.74	69.8	30.2	74.9	25.1	90.6	9.4
	4	2210	3.67	72.0	28.0	77.2	22.8	90.3	9.7
	x	1800	3.99	70.4	29.6	75.2	24.8	88.7	11.3
	y	2100	3.80					90.6	9.4
	z	2050	3.76					90.2	9.8
Average.....		2153	4.14	72.1	27.9	76.9	23.1	90.3	9.7
Fasted 48 hrs.	1	2250	3.17	75.2	24.8	78.4	21.6	92.4	7.6
	2	1770	2.53	69.8	30.2	73.6	26.4	90.1	9.9
	3	1450	3.12	66.8	33.2	71.8	28.2	89.5	10.5
	4	1960	3.20	68.1	31.9	73.7	26.3	88.1	11.9
	*	2150	2.25	76.8	23.2	82.2	17.8	88.3	11.7
Average.....		1916	2.85	71.3	28.7	75.9	24.1	89.7	10.3
Fasted 5 days	1	1509	2.83	75.9	24.1	78.1	21.9	92.1	7.9
	2	1500	2.87	74.2	25.8	77.9	22.1	91.3	8.7
	3	1550	3.02	74.3	25.7	80.3	19.7	90.3	9.7
	4	1600	2.63	72.6	27.4	75.5	24.5	89.5	10.5
	5	1750	2.75	72.8	27.2	76.6	23.4	89.1	10.9
Average.....		1582	2.82	74.0	26.0	77.7	22.3	90.5	9.5
Average of all.....				73.6	26.4	77.7	22.3	90.4	9.6

* Fasted 3 days.

ligature was then cut away. After severing the ligaments attaching the "main liver" to the diaphragm, a ligature was placed about it. This ligature, tied just ventral to the diaphragm without inclusion of the vena cava, permitted excision of the "main liver."

In some instances interference with the circulation of the remaining liver tissue resulted in necrosis of these parts. Rarely the vena cava was obstructed by the ligatures about the "posterior lobe mass" and very rarely a bile leak or hemorrhage occurred. Occasionally, too, animals developed a postoperative pneumonitis. Such instances have been ruled out in considering the evidences of liver insufficiency induced by this method. They have amounted in all to about 15 per cent.

After excision of nine-tenths of the liver tissue, in the rabbit the remaining portions appeared slightly congested but never turgid and tense as they do after the removal of but three-fourths of the organ in dogs and rats (11, 13). The venous channels became moderately dilated and a fatty infiltration of the parenchyma developed. Furthermore changes indicative of a fatty degeneration were found histologically, this serving to increase further the degree of liver insufficiency obtained by the operative procedure. Autopsy yielded no evidences of severe portal obstruction. There was no pronounced dilatation of the portal tributaries, and no marked congestion of the gastric or intestinal mucous membranes or engorgement of the spleen. Even the remaining portions of liver were not greatly distended with blood. Nevertheless we have asked ourselves, Can death have been due to obstruction to the flow of portal blood occasioned by the operation? To answer this question a series of control experiments were carried out in which an equivalent or greater obstruction of the portal blood flow was induced with but a negligible removal of liver parenchyma.

Control Experiments.

In seven rabbits, the portion of the "posterior lobe mass" of the liver lying to the right of the vena cava was removed under ether anesthesia as in the operation for nine-tenths liver ablation. The portal vessels to the "main liver" were then ligated, leaving intact all branches of the bile ducts and the hepatic artery. In this way the portal blood to 90 per cent of the liver was shunted through the caudate lobe and the remaining stump of the "posterior lobe mass." 4 to 8 days later, India ink was injected into the portal vein of the surviving rabbits to determine how great an amount of portal obstruction had actually been obtained. In

five instances the ink flowed only to the 10 per cent of liver which, according to the conditions of the experiment, should have received portal blood. In the two remaining rabbits some ink reached the main liver, showing that the portal blood had some access to it.

These animals in which a diversion of 90 per cent of the portal blood to the liver had been successfully effected had all survived in good condition.

In another group of four rabbits the branch of the portal vein which leads to the "posterior lobe mass" was ligated following ablation of the "main liver." This procedure caused an even greater blocking of the portal blood flow than that above described; yet the animals did not die until about 5 days after the operation. The sole road for the portal blood had been through the small caudate lobe. Marked hypertrophy of this lobe was found with dilatation of its channels and one may infer that obstruction to the portal flow had progressively diminished from the time of the operation. Death was preceded by the typical signs of liver insufficiency, to be outlined below.

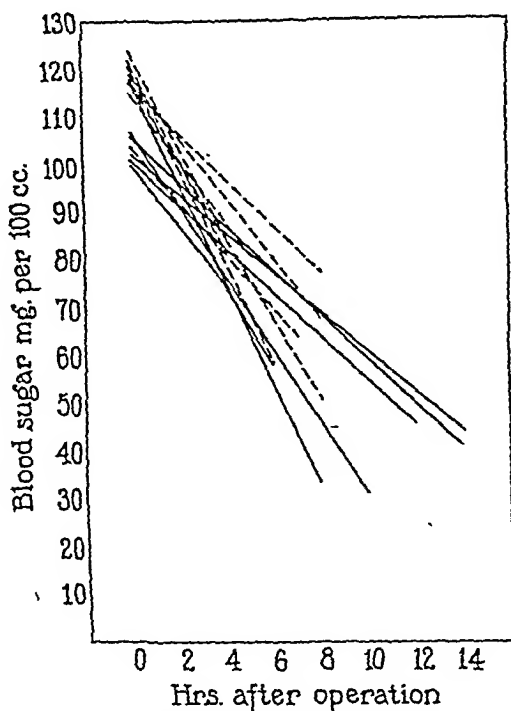
It is evident that death of rabbits when 90 per cent of the liver has been removed by our method cannot be ascribed to the portal obstruction incident to the operation. As will be reported in a succeeding paper, we have found it possible to occlude in the rabbit from 9/10 to 19/20 of the lumen of the portal vein together with 4/5 to 9/10 of the lumen of the vena cava without jeopardizing the animal.

The Evidences of Liver Insufficiency after Ablation of 90 Per Cent of the Rabbit Liver.

The Fall in Blood Sugar.—Following removal of nine-tenths of the liver the animals made a rapid recovery from the anesthetic, drank water, and appeared to be in excellent condition. In a few hours, however, they were to be found with drooping head, sprawling legs, and obvious muscular weakness. The heart rate was rapid, rising to 285 beats per minute from a preoperative average of about 140. In 6 to 12 hours the animals became prostrate and convulsions supervened, in one of which death occurred about 8 to 18 hours after operation. Mann and Magath have described (2, 3) a rapid fall in the blood sugar concentration of dogs deprived of the entire liver, with result in convulsions and death a few hours after the operation. So too in our rabbits deprived of 90 per cent of the organ the blood sugar fell rapidly to a level below the minimum compatible with life.

In numerous instances we have followed the blood sugar concentration in rabbits before and after removal of nine-tenths of the liver,

employing for the purpose the Hagedorn-Jensen method (18, 19). Text-fig. 1 shows the rapid decrease of blood sugar resulting from the liver loss, in fourteen of these animals. In the instances depicted by the solid lines, the blood sugar concentration fell to a level incompat-



TEXT-FIG. 1. *Fall in the Blood Sugar Concentration in Rabbits Following Ablation of 90 Per Cent of the Liver.*

The blood sugar findings in fourteen rabbits deprived of 90 per cent of the liver are plotted in terms of milligrams of glucose per 100 cc. of blood. In all the animals a rapid fall in the amount of blood sugar occurred. In the instances depicted by continuous lines, typical hypoglycemic symptoms developed and the animals died. The dotted lines show that a similar fall in blood sugar concentration took place in eight animals, until they were given glucose at the points where the lines are discontinued.

ible with life; convulsions and death followed. The dotted lines show an equally rapid initial fall in the blood sugar of other rabbits, which was checked by injections of glucose given at the points indicated by the discontinuance of the lines.

The Insufficiency in Rabbits Given Glucose after Ablation of 90 Per Cent of the Liver.

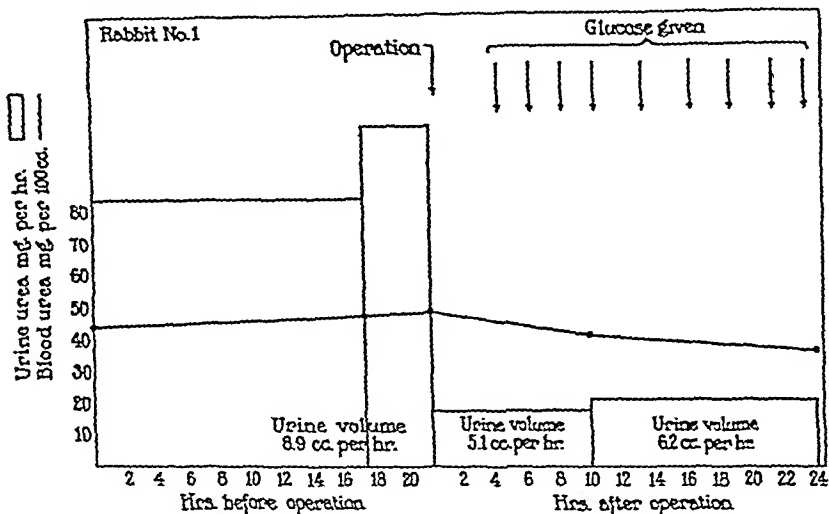
Mann and Magath showed (3) that administrations of glucose to dogs deprived of the total liver preserved life for many hours. Eventually the animals died, in spite of the maintenance of a high blood sugar level, in a condition characterized by weakness and flaccid paralysis. The same phenomenon more prolonged was found in our rabbits given glucose after ablations of about 90 per cent of the liver. The substance was usually administered by stomach tube, but often by intravenous injection, and at times subcutaneously, in varying doses and at various intervals of time.

The effect of glucose, especially by intravenous injection, in rabbits deprived of 90 per cent of the liver is as startling as in completely hepatectomized dogs (3, 9). Prostrated, moribund animals will be found sitting up or moving about freely a few minutes after a single injection of 10 to 20 cc. of 5 or 10 per cent dextrose solution. The pulse and respiratory rates are slowed and the animal appears normal again. The effect, however, is not long lived. 30 minutes to several hours later the rabbit again becomes weak, the pulse and the respiration rate are increased, the blood sugar is low, reflexes are exaggerated, and convulsions may occur. Again and again the animal may be restored by administrations of glucose but finally, in spite of these, death comes on, heralded by extreme asthenia, coma, and respiratory failure.

Derangements in Urea and Uric Acid Metabolism.—It is now well known that urea formation ceases in the liverless dog (20). This phenomenon is accompanied by an accumulation of uric acid in the blood, with a consequent increased excretion of the substance if kidney function is maintained. The change from a normal urea metabolism does not appear in dogs deprived of as much as 70 per cent of the liver after an Eck fistula operation (9, 21), although uric acid destruction may possibly fail to occur. Indications of a lack of urea formation or of cessation of uric acid destruction in dogs may be taken as definite evidence of liver insufficiency (9). Such evidence has been obtained in our partially hepatectomized rabbits.

Procedure.—Freshly fed rabbits were fasted for 24 hours, given 50 to 75 cc. of 5 per cent glucose solution by gavage, and later allowed to drink the same solution

at will. A copious secretion of urine resulted. 12 to 20 hours later the animals were catheterized, the bladder washed thoroughly with water, and a blood sample taken from an ear vein. They were then placed in clean metabolism cages for 17 to 24 hours. At the end of this period another blood specimen was taken, and the catheterization and bladder washing repeated. The urine thus obtained and that collected during the previous 17 to 24 hour period was analyzed for urea and uric acid. At once after taking the second catheter specimen 90 per cent of the liver was removed under ether anesthesia, and for varying periods thereafter

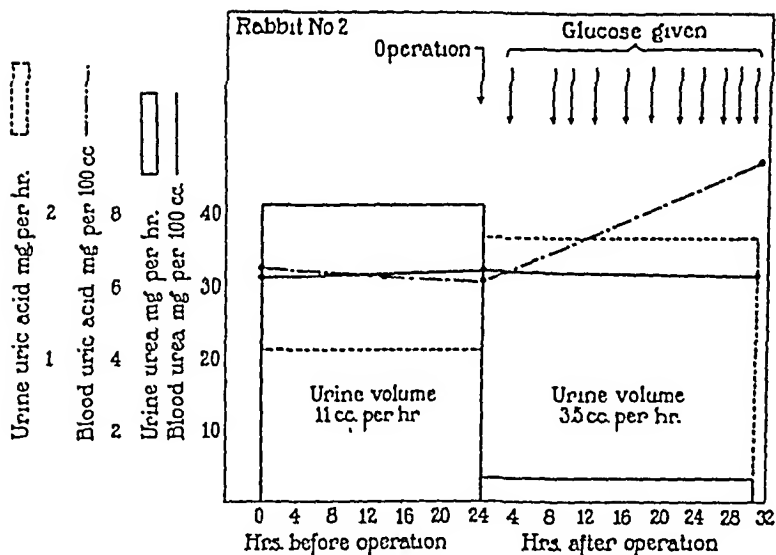


TEXT-FIG. 2. Changes in Blood Urea Concentration and Urea Output in the Urine after Removal of 90 Per Cent of the Liver.

Uric acid studies were not made. The changes shown are similar to those described in Text-figs. 3 to 6.

the animals were kept in metabolism cages. From time to time they were given water or 5 per cent glucose solution to maintain diuresis. At intervals they were catheterized and the urine and bladder washings added to the cage urine specimens to be analyzed for uric acid and urea. Blood specimens taken during or immediately before the catheterizations were used for blood urea and blood uric acid analyses. For the determinations of urea concentration in blood and urine, the method described by Addis (22) was employed. In some instances (Text-figs. 2, 4, and 5) the technique of Van Slyke (23) was used as well. Folin's method (24) served for the estimation of uric acid in the blood, and the procedure of Folin and Wu (25) for its determination in urine.

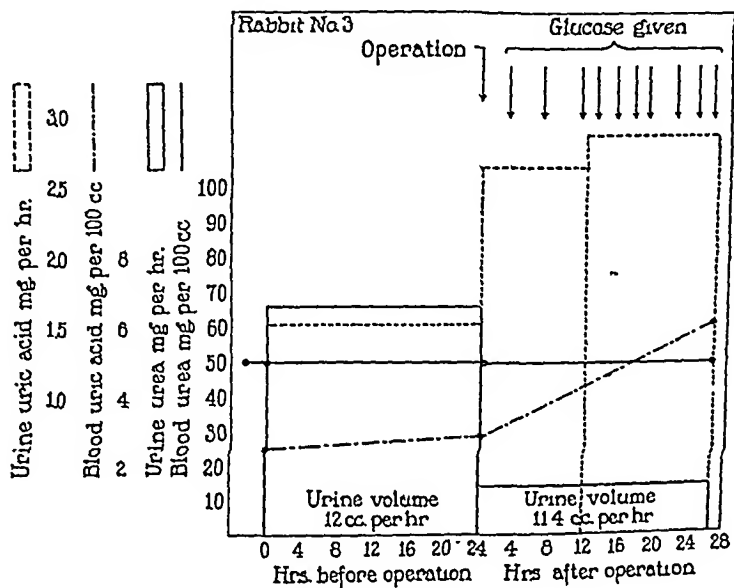
In all five instances depicted in Text-figs. 2 to 6 adequate diuresis was maintained. In all a striking decrease of urea output in the urine



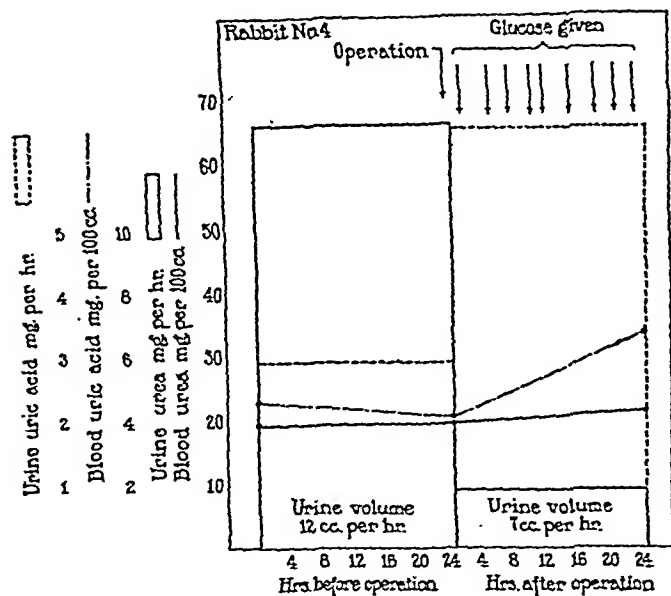
TEXT-FIG. 3.

TEXT-FIGS 3 to 6. *Changes in the Urinary Output and Blood Concentration of Urea and Uric Acid in Rabbits Deprived of 90 Per Cent of the Liver.*

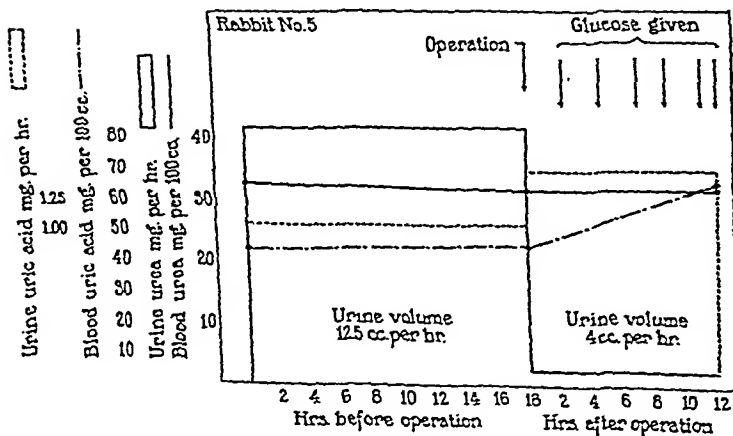
Text-figs 3 to 6 depict the uric acid and urea findings in the blood and urine of rabbits before and after ablation of 90 per cent of the liver. In all, the urea output of the urine decreased profoundly after the ablation while the blood urea concentration remained almost unchanged. In all, an increased urinary output of uric acid appeared, together with a greater concentration of the substance in the blood.



TEXT-FIG. 4.



TEXT-FIG. 5.



TEXT-FIG. 6.

occurred. Despite this lack of urea output the blood urea concentration remained unchanged in three of the animals, fell in one (Text-fig. 2), and rose but slightly in two, affording thereby evidence of greatly decreased urea formation. The uric acid studies made on four of these animals, Text-figs. 3 to 6, showed an increase of this substance in both blood and urine after ablation of 90 per cent of the liver.

In considering the evidences of liver insufficiency in these animals it is to be noted too that a mild tissue icterus appeared, accompanied by an output of bile pigment in the urine, as shown by the Van den Bergh test.

Liver Insufficiency in Rabbits Deprived of 80 Per Cent of the Liver.

Similar studies were made on three animals deprived of but 80 per cent of the liver. The findings were not clear-cut. The almost complete absence of urea excretion observed after ablation of 90 per cent of the liver was not present in these instances. In two of the animals the urinary output of urea per hour amounted to about half that of the preoperative period, while in the third animal no decrease was observed. Despite this the blood urea concentration of all rose notably, yielding evidence of a normal formation of urea in the case of the animal last mentioned, and of only a slight decrease in the others. In but one of the three experiments was an increase in urinary uric acid noted and this appeared in the instance showing normal urea formation. From these findings one might suppose that by removal of 80 per cent of the organ a borderline condition had been obtained in which there might or might not be liver insufficiency, in respect to these functions.

The Degree of Liver Insufficiency in Rabbits Following Ablation of 70 Per Cent of the Organ.

Prior to the adoption of the method described above, the "main livers" of several rabbits were ablated in the hope that the animals would develop a true liver insufficiency, for the operation entails a loss of 70 per cent of the organ. In 1889 Ponfick (16) reported his results with this procedure, stating that practically all the animals died. Of twenty-one rabbits operated upon in our series, seven survived in

good health for several weeks, when they were autopsied. Two others died of postoperative pneumonia and five others lived about 6 days, succumbing from undetermined causes. In the remaining seven instances, death occurred in about 20 hours accompanied by very low blood sugar concentration and convulsions. Ordinarily one finds after removal of the rabbit's "main liver" a transient fall in the blood sugar which may be sufficient to bring about death. The other evidences of liver insufficiency, the clinical picture of asthenia and prostration with faulty urea and uric acid metabolism, are in our experience lacking.

DISCUSSION.

The evidence presented herein clearly shows that a fatal liver insufficiency develops in rabbits deprived of 90 per cent of the organ. Following this great reduction in parenchyma the blood sugar falls to the lethal point. If this be avoided by the administration of dextrose the insufficiency manifests itself in a partial failure of the organ to form urea and to transform uric acid. Eventually the animal dies, of causes unknown.

After ablation of 90 per cent of the liver a mild jaundice appears, showing that there is a deficiency in the function of bile elimination. Earlier work from this laboratory, on the biliary obstruction required to produce jaundice in dogs, is in agreement with this finding. McMaster and Rous showed that jaundice of biliary obstruction does not appear after ligating several of the branches of the hepatic duct unless the drainage from 95 per cent or more of the liver is obstructed (12).

The fact is important that the rabbit deprived of 90 per cent of its liver behaves like the liverless dog in all ways that have thus far been tested. The blood sugar concentration falls, convulsions occur, urea formation ceases, uric acid metabolism is disturbed, and even when the blood sugar is artificially maintained at a high level death occurs, with symptoms similar to those arising in the liverless dog (9).

SUMMARY.

A rapid and simple method for the production of marked liver insufficiency in rabbits has been described. The necessary operation can be carried out by an unassisted operator in a few minutes. The method should further the study of liver physiology.

The changes as concerns blood sugar, urea formation, and uric acid metabolism would appear to be the same in the rabbit suffering from hepatic insufficiency as in the dog.

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TOTAL SURGICAL REMOVAL OF THE LIVER IN RABBITS.

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Total surgical removal of the liver in the rabbit without impairment of the portal and caval circulation has not hitherto been reported. A method has been devised for the purpose of work in this laboratory. It would seem to be desirable to report its technique not only because of the wide use of the rabbit as an experimental animal but because of the frequency with which it has been employed in studies upon the relation of the liver to carbohydrate metabolism (1).

Excision of the liver necessitates occlusion of the portal vein. Moreover the intimate attachment of the organ to the vena cava requires the removal of a segment of this vessel as well, if a complete and satisfactory liver ablation is to be obtained. In the normal rabbit the obstruction of either, or both, of these veins is followed by death so rapidly that in a method for hepatectomy channels must be provided for the immediate return of caval and portal blood to the heart. Markowitz and Soskin (2) have reported a technique for inducing a collateral circulation in dogs without an Eck fistula. At a preliminary operation the portal vein and vena cava are partially occluded with ligatures which constrict the lumina of these vessels to about one-fifth the original size. After some weeks collaterals to the veins are so well developed that the liver may be removed without impairment of the portal circulation. The present method is a modification of this technique.

Our first attempts with the rabbit were unsuccessful. The vena cava and portal vein had been approached directly, and exposed on the right side of the abdomen. The consequent injury to the ventral surface of the liver and the peritoneum of this region resulted in massive adhesions among the injured parts, preventing removal of the liver without great blood loss. It therefore became imperative to devise a method whereby these veins could be approached, and

partially ligated, from the left side of the abdomen, without exposing the liver, leaving the right side free of adhesions that would hinder the later hepatectomy.

Anatomical Peculiarities of the Rabbit's Liver.

The liver of the rabbit consists of two relatively separate masses connected by a thin isthmus of parenchyma. The larger mass, comprising the three cephalad lobes, has been termed the "main liver" and the smaller portion, the "posterior lobe mass" (3). Each possesses its own arteries, ducts and branches of the portal vein. The main portal trunk on approaching the liver divides to send its first branch to the "lobe mass." At about this level it also receives a tributary—the superior pancreatico-duodenal vein—and in this region too the hepatic artery comes to lie close to the vessel and accompany it to the liver. Still another tributary, the left gastro-epiploic, enters the portal vein about 1 cm. cephalad to the juncture of the portal and superior pancreatico-duodenal veins. The relationship of these structures is by no means constant. Thus, for example, the superior pancreatico-duodenal vein may enter the portal stream either below or above the great branch of the portal to the "posterior lobe mass" of the liver. As the aim of the preliminary operation is the establishment of a portal obstruction so great that it will induce the development of a collateral circulation, it is necessary to produce this obstruction caudad to the first branch of the portal vein, that to the "lobe mass." Unfortunately the tributaries mentioned above, the superior pancreatico-duodenal and the left gastro-epiploic, usually enter the portal vein above this site of election and, as our experience has shown, will serve as by-passes, whereby portal blood reaches the "main liver." To cut off this source of supply it is necessary to ligate the two small vessels individually.

The Preliminary Operation.

The shaved skin of the rabbit is swabbed with 60 per cent alcohol and under ether anesthesia the abdominal cavity is exposed from the level of the ensiform to the umbilicus by an incision 1 cm. to the left of the mid-line. The relatively bloodless mid-region is thus left intact for the incision of the secondary operation. The stomach is pressed upward, covering the liver, and the portal vein is approached from the left side and sufficiently freed of the surrounding tissue for a

silk ligature, soaked in petrolatum, to be passed about it. The ligature is placed just caudad to the branch to the "posterior lobe mass" and to the junction of the superior pancreatico-duodenal and portal veins. Great care should be exercised to place the ligature around the vein only, leaving intact between it and the liver the peritoneum adherent to its right side, thus preventing all contact between silk and the liver. Only if this is successfully accomplished will adhesions fail to develop.

The ligature is tied down so as to include not only the vein but the end of a glass rod. This end is bent at a right angle to the main shaft and is laid parallel to the vessel for ease in tying. It should taper slightly so that it can be readily withdrawn after the tie has been accomplished leaving the vein partially ligated, with a lumen equal to the size of the rod. The diameter of the latter should be 2 mm. for a 2 kilo animal.

The superior pancreatico-duodenal vein is tied near its junction with the portal, and likewise the small vein which arises on the caudad surface of the pylorus and leads into the portal about 1 cm. above the entrance of the superior pancreatico-duodenal vein. At times this vessel may enter the portal vein just dorsal to the pylorus. Both these procedures, carried out without injury to the peritoneum of the right side of the abdomen, are essential; for as already mentioned these veins, unless occluded, enlarge with extreme rapidity and soon provide an adequate supply of blood to the liver. The ligation of the small left gastro-epiploic vein can be carried out only by a dissection along the posterior wall of the pylorus. The vein receives, as a rule, one or more tributaries. It should be inspected along its entire course and the ligature placed below the last tributary, a delicate procedure which exposes the bile duct and portal vein in this region as well.

Finally partial occlusion of the vena cava is effected from the left side of the abdomen. As a first step the superior mesenteric artery is identified and the peritoneum perforated on the left side of the mesentery just cephalad to this artery and just ventral to the aorta. A small glass spatula covered with vaseline is introduced into the aperture and worked between the vessel and the peritoneum anterior to it. A threaded ligature passer introduced along the track thus made is turned posteriorly about the vena cava, thus carrying a ligature around the vessel, just caudad to the right adrenal body. The lumen of the vena cava is then reduced to 2 mm. by tying the ligature about the removable glass rod as in the case of the portal vein.

Recovery from the operation is usually rapid and a collateral circulation soon develops about the liver. In a few days, if the procedure has been successful, evidences of this fact appear, in a notable enlargement of the veins of the abdominal wall. The development of this collateral circulation is so rapid that one can totally occlude the portal vein and the vena cava 5 days after the primary operation without the appearance of any untoward symptoms. This has been done in 4 instances as a control experiment to demonstrate the existence of an adequate by-pass for the blood about the liver. In our later work, however, we have gener-

ally allowed an interval of 3 weeks to elapse between the primary and secondary operations. This not only affords ample time for the development of the new vascular bed about the liver but suffices for the animal's complete return to general health. Liver extirpations have been done successfully as late as 6 months after the preliminary operation.

Hepatectomy in the Rabbit.

The incision for hepatectomy in the rabbit should run in the mid-line from ensiform to umbilicus. A ligature is placed around the entire gastrohepatic omentum, including in the one tie the portal vein, bile duct and hepatic artery. The vena cava is ligated just cephalad to the right adrenal gland, and again just cephalad to its union with the hepatic veins from the "main liver," and severed between. The structures in the gastrohepatic omentum are cut cephalad to the tie placed about them. The liver can now be removed *in toto*, after cutting its ligaments to the diaphragm and dividing the peritoneum on either side of the vena cava in the regions where this is intimately attached to the liver. No stump of hepatic tissue need be left.

A diaphragmatic vein enters the right side of the vena cava slightly caudad to the entrance of the hepatic veins from the "main liver." This small vessel must be tied and cut between ligatures.

In certain instances it may be of advantage to remove the "main liver" early in the operation, immediately after tying the gastrohepatic omentum, thereby gaining working space for the more difficult steps. This is done by snipping the ligamentous attachments between the "main liver" and diaphragm and throwing a stout ligature around the former, tying it down closely upon the vena cava. The "main liver" is then cut away above this ligature. In the final stage of the operation, its stump is removed.

DISCUSSION.

The clinical picture in the rabbit, deprived of the entire liver, needs only brief mention for in all important particulars it is like that in the liverless dog, described by Mann (4). As is now well known, hepatectomy in the dog is followed by pronounced hypoglycemia with an accompanying train of typical symptoms. Further, this condition fails to appear when the diminution of blood sugar is prevented by administrations of glucose. The postoperative state in the liverless dog may be divided into two clearly defined stages, the first characterized by the hypoglycemic symptoms, the second, even if glucose be given, by coma terminating in death.

All these phenomena have their counterpart in the rabbit, as our observations upon these animals deprived of 90 per cent of the liver

(5) have already shown. Completely hepatectomized animals treated by the administration of dextrose in 25 per cent solution *per os* or in 5.4 per cent solution intravenously in sufficient amounts to keep the blood sugar concentration at or about 125 mg. per cent behave normally for from 12 to 32 hours. In this first stage no untoward symptoms appear. As a rule only slight variations of the pulse and respiratory rate are noticed, together with an increase in body temperature of 1 to 1.5°. Finally, in spite of the glucose administrations, certain phenomena usher in what may be called the second stage. Now the animal when placed on the floor no longer investigates its surroundings in the ordinary way but hops blindly in a straight line, striking any object in its path, even a brilliantly lighted wall. Later on evidences of muscular weakness and ataxia appear; the animal sits with sprawling legs and drooping head. Left in the cage it will be found with nose pressed in a corner. In the next few hours, although care is taken that the blood sugar level does not fall below normal, the weakness progresses and the movements become more ataxic. The animal falls to one side in attempting to move and cannot rise again, or it lies with sprawling legs unable to raise its head or draw the limbs under the body. The temperature becomes subnormal, the respiratory movements are labored and less frequent, and the pulse is slower too. The corneal reflex and knee jerk previously present are lost and complete flaccid paralysis, save of the respiratory muscles, sets in. For a period Cheyne-Stokes respiration may occur, after which the respiratory rate falls to 8 per minute or even lower, and while the pulse rate remains at about 30 per minute the heart sounds progressively weaken. After varying periods up to 40 hours death comes on, with respiratory failure, the heart continuing to beat for 10 minutes or more after respiration has ceased.

At the present time we can venture no explanation of the later symptoms. It is of importance now merely to call attention to the close similarity of the postoperative events in the liverless rabbit and dog, a similarity which permits us to infer perhaps that the major functions of the organ are much the same though the one creature is herbivorous and the other omnivorous.

SUMMARY.

A technique is described for total removal of the liver of the rabbit without circulatory difficulties as a result. The method requires a preliminary operation to induce a development of portal and caval collaterals.

Rabbits deprived of the liver in this manner if given glucose live for varying periods up to 40 hours. Before death they show the same disturbances as do hepatectomized dogs. They die early, of hypoglycemia, unless provided with sugar.

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THE RELATION OF THE LIVER TO FAT METABOLISM.*

I. EFFECT OF LIVER LACK ON FAT COMBUSTION AND THE RESPIRATORY QUOTIENT.

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It is generally agreed that the liver is active in fat metabolism in many ways. As early as 1886 this became evident to Nasse (1). Subsequent systematic studies by Noel Paton (2), Leathes (3, 4), Leathes and Raper (5), Raper (6), Mottram (7), Rosenfeld (8), Bloor (9, 10, 11) and others, have resulted in a mass of evidence, demonstrating various liver activities in the metabolism of fat. But are these functions performed solely by the liver and are they vital to the organism? Can fat combustion be carried on at all in the absence of the organ? These are the chief questions concerning the rôle of the liver in fat metabolism. Their answer waits upon a demonstration of the absence or continued presence of fat combustion in animals deprived of the liver or otherwise brought into a state of extreme hepatic insufficiency. In the present communication we will report experiments upon this theme. Rabbits were chosen for the work and total liver ablations done (12) by a method already described. For the induction of a partial but pronounced liver insufficiency, removal of approximately 90 per cent of the liver was practiced (13).

The Respiratory Quotient of Rabbits with Partial Liver Insufficiency.

Earlier workers have shown that the respiratory quotient of fasting animals, previously well fed, is low (14), indicating a body metabolism largely that of fat. What now will the quotient be after removal of the liver, or in conditions of hepatic insufficiency?

* A preliminary report upon some of the findings given herein has already appeared in the *Proceedings of the Society for Experimental Biology and Medicine*, 1927, xxv, 151.

Well nourished rabbits on a mixed diet, and weighing 2 to 3 kilos, were fasted 48 to 72 hours and subjected to a preliminary determination of the respiratory quotient by the method outlined below. The respiratory quotient was low in such fasting animals indicating a pronounced combustion of fat. Then liver insufficiency was induced.

Method.

The Respiratory Quotient. The Apparatus.—All respiratory quotient determinations were made by the closed circuit method with the rabbits sealed in an air tight respiration chamber, a very large desiccator, submerged in a constant temperature bath. The chamber was just large enough to allow the animals the normal crouching posture. The closed circuit consisted of the animal respiration chamber, two parallel absorbing systems with shut-off clamps, a mercury pump which afforded a continuous circulation of air, and a set of valves permitting the air to pass through the circuit in one direction only. The total volume of the apparatus was 11 liters. The absorbing systems, for the removal of water vapor and CO₂, were each made up of an ascarite¹ tube connected at both ends with sulfuric acid absorption bottles. The weight of the ascarite tube in one of these systems was carefully determined, and so too was that of the second sulfuric acid bottle which the air entered after passing through the ascarite. The circulating air could be shunted at will through either of these absorption circuits. From a reservoir of known volume, oxygen entered the closed circuit between the absorbers and the pump. At this point too, connections were made with an auxiliary oxygen reservoir. At frequent intervals the apparatus was tested for leaks, by raising the pressure by an amount equal to that of 100 cm. of water in addition to the atmospheric pressure and ascertaining that no volume change occurred in the circuit within 5 minutes.

The Respiratory Quotient Determination.—The respiration chamber containing the experimental animal was submerged in the constant temperature bath and the air within the system circulated at the rate of 3 liters a minute. But one of the absorption systems was employed in the circuit and oxygen was used from the auxiliary oxygen reservoir. After 20 minutes the pump was stopped and the pressure within the system adjusted to that of the room atmosphere. The barometric pressure was noted at the same time. Upon starting the circulation of air again the current was deflected through the second train of absorbers containing the weighed ascarite tube and weighed sulfuric acid adsorption bottles. Oxygen was now allowed to enter the closed system from the measured reservoir until about 1.5 liters had been consumed. This required about 1 hour and 20 minutes with the average 2 to 2.5 kilo rabbit. At the end of this period the pump was

¹ "Ascarite," a mixture of asbestos and sodium hydroxide, distributed by A. H. Thomas Company, Philadelphia.

stopped, the pressure in the closed system adjusted again to that of the room atmosphere, the barometric pressure observed, and the volume of oxygen added to the system during the period was noted. By reweighing the ascarite tube and second sulfuric acid bottle the amount of CO_2 produced was determined. The volume of oxygen added during the period of the experiment, plus or minus the correction for changes in barometric pressure, temperature and vapor pressure, represented the oxygen consumed.

The rabbits remained quiet within the respiration chamber. It was not necessary to prevent all motion on the animal's part for calorimetry studies were not contemplated and only the gas exchange was measured. However the animals sat so tranquilly that we were able, in later work, to give them continuous intravenous injections of glucose while in the chamber, despite the fact that the injecting needle once placed in an ear vein could not be readjusted during the period of the respiratory quotient determination. In over 20 such experiments no animal ever moved sufficiently to dislodge the needle from the vein. The method will be described further on.

Technique.—When it had been established that the respiratory quotient of a fasted rabbit was definitely low, indicating that fat combustion was taking place, blood specimens were taken from an ear vein for sugar, hemoglobin and hematocrit estimations, and immediately thereafter 90 per cent of the liver was ablated under ether. 5 to 7 hours later, when the immediate effects of operation and anesthesia had worn off, a second determination of the respiratory quotient was made. Immediately preceding the animal's entrance into the respiration chamber and at once following its removal therefrom, samples of venous blood were taken by cardiopuncture from the right ventricle for CO_2 and sugar analyses.

As we have shown in a preceding paper the blood sugar concentration falls rapidly after removal of 90 per cent of the liver, reaching the lethal minimum 6 to 10 hours after the operation. As the second respiratory quotient determinations were made in the latter part of this period the blood sugar level was invariably found low, between 60 and 70 mg. per cent. Often the animals collapsed and became moribund while in the respiration chamber the second time. In such instances the experiments were discontinued.

In four instances a third respiratory quotient was determined on the following day, 24 hours or more after the removal of 90 per cent of the liver. These animals, of course, received glucose during the night in amounts just sufficient to keep the blood sugar level slightly above the minimum required to keep them free from convulsions. Blood sugar analyses were made before the final respiratory quotient was taken to rule out instances in which the giving of too much glucose might have brought the blood sugar concentration to normal or above. It seemed conceivable in this event, indeed it has been shown by our later work, that the utilization of glucose by the animal might mask or entirely supplant the combustion of fat. In one instance, Table I, No. 4, the blood sugar concentration was found high (0.099 per cent) on the day following operation and the respiratory quotient determination was duly postponed several hours, until it had fallen to 0.079 per cent.

TABLE I.

Respiratory Quotient, Metabolic Rate and Blood Sugar Findings in Rabbits Deprived of 90.6 Per Cent of the Liver.*

No.	Preoperative findings			6 to 8 hours after operation			24 hours after operation		
	Respira- tory quotient	O ₂ con- sumption	Blood sugar	Respira- tory quotient	O ₂ con- sumption	Blood sugar	Respira- tory quotient	O ₂ con- sumption	Blood sugar
		mg. per min.	mg. per cent		mg. per min.	mg. per cent		mg. per min.	mg. per cent
1	0.740	27.5	0.114	0.720	27.8	0.064	—	—	—
2	0.766	20.7	0.119	0.757	18.5	0.069	—	—	—
3	0.783	19.3	0.118	0.720	18.9	0.077	—	—	—
4	0.729	28.0	0.122	—	—	—	0.731	32.6	0.079
5	0.782	33.6	0.128	—	—	—	0.745	31.9	0.056
6	0.768	30.0	0.125	—	—	—	0.735	29.5	0.046
7	0.760	25.5	0.122	0.739	25.0	0.059	0.743	26.1	0.066
8	0.826	29.9	0.128	0.751	27.7	0.085	0.714†	29.6	0.051

* Expressed in terms of O₂ consumption per minute.

† 10 hours after operation.

TABLE II.

Blood CO₂ of Rabbits Immediately before and after the Period of Respiratory Quotient Determination.

No.	Before entering respiration chamber	After entering respiration chamber	Hours after operation
	vol. per cent	vol. per cent	
1	48.1	48.3	6
2	46.8	47.6	6
3	39.6	39.9	6
4	47.5	47.2	24
5	40.1	40.5	24
6	42.7	43.1	26
7	44.4	44.4	5
7	41.6	40.6	24
8	46.0	45.6	6
8	45.8	45.5	10

As an additional routine measure, blood specimens from the ear vein were taken before operation and at variable intervals thereafter for sugar estimation by the Hagedorn-Jensen (15) method. After operation, blood specimens for CO₂ analysis were taken immediately preceding, and at once following the sojourn of

the animals in the respiration chamber. They were obtained by cardiopuncture of the right ventricle, under oil, and in paraffined tubes. The CO_2 estimations were carried out by the method of Van Slyke and Sendroy (16, 17). These latter findings for the instances shown in Table I are presented separately in Table II. As the respiration chamber was small and the animals quiet, differences in the metabolic rate could be roughly determined in terms of oxygen consumption per minute.

Only those instances have been considered in which the metabolic rate remained constant before and after operation (see Table I). All experiments have been ruled out, too, in which any significant change in the concentration of blood CO_2 was found. The results given below must be attributed to the true gas exchange of the animal and not to mere retention or blowing off of CO_2 during the periods of experimentation.

In these experiments, which were of relatively brief duration, no significant hemoglobin or hematocrit changes were observed.

Findings.

The preliminary respiratory quotients of 25 fasted rabbits averaged 0.755, with variations between 0.722 and 0.826, indicating a great combustion of fat. In Table I the respiratory quotient, blood sugar and oxygen consumption data are given in 8 of these instances which constitute experiments free from objection, in which neither blood CO_2 changes nor significant variations in the metabolic rate appeared. The magnitude and character of the individual variations are self-evident. When about 90 per cent of the liver of the animals was ablated, the respiratory quotient 6 to 8 hours later averaged 0.737, with individual variations between 0.720 and 0.757. The respiratory quotients of 4 of these rabbits taken again the following day averaged 0.738, with only slight variations,—0.731 to 0.745.

In 4 other successful experiments, not shown in the table, the respiratory quotient and blood sugar findings were similar but the metabolic rate showed some retardation, the animals requiring $1\frac{3}{4}$ to 2 hours, even $2\frac{1}{2}$ hours in one instance, to consume the quantity of oxygen used prior to operation in about $1\frac{1}{4}$ to $1\frac{1}{2}$ hours. These latter instances are merely corroborative and hence need be mentioned no further.

From the findings reported so far we conclude that fat combustion

can be carried on as readily and as rapidly in the rabbit after ablation of 90 per cent of the liver as before, in spite of the fact that the animals suffer from extreme liver insufficiency, eventually dying therefrom (13).

The Respiratory Quotient of Rabbits Deprived of the Entire Liver.

With these facts established, it became essential to determine whether fat combustion could continue in the absence of the entire liver. In the experiments just described, there was the possibility that the findings depended upon some activity of the small remnant of the liver. For this reason we repeated the experiments, as described above, but employed rabbits deprived of the entire organ according to the method described in an earlier paper (12).

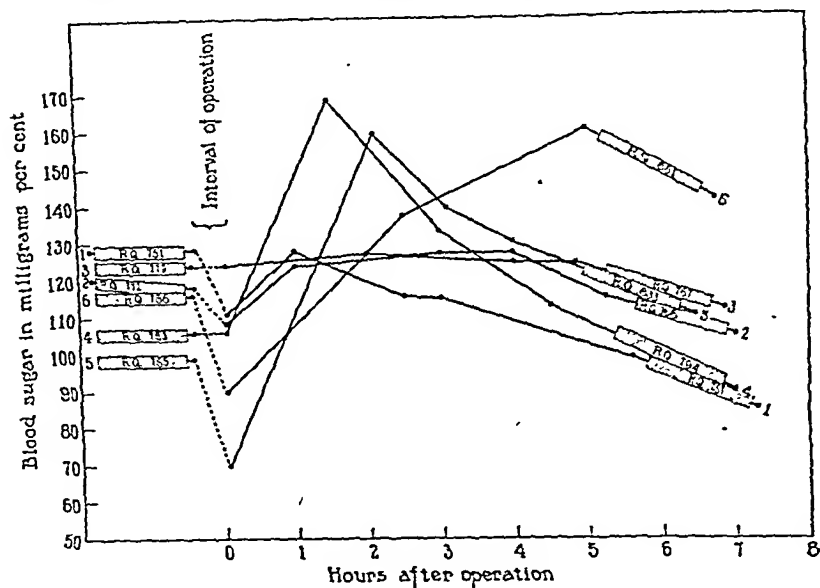
One important change in technique was forced upon us. The rabbits deprived of the entire liver required glucose prior to the postoperative estimation of the respiratory quotient. For the fall in blood sugar concentration took place more rapidly in them than in those animals retaining 10 per cent of the organ. In the liverless rabbit given no glucose death from low blood sugar occurs in some instances within 2 to 3 hours after operation; and it seemed wise to wait at least 4 hours before determinations of the respiratory quotient since both metabolism and respiratory quotient might have been disturbed by the ether anesthesia and laparotomy. Preliminary experiments were made to determine the minimum amount of glucose which would maintain hepatectomized rabbits. These experiments will be detailed in a following communication. Suffice it to say here that 100 to 130 mg. of glucose per kilo of body weight per hour answers the purpose, when given by continuous injection during the first 8 hours after hepatectomy. This small amount of glucose only was given in the later work, since a larger dosage might conceivably have acted to mask an existing fat consumption.

Technique.

For the respiratory quotient experiments a technique was used similar to that just described, with certain minor modifications.

Rabbits of about 2 kilos were subjected to subtotal ligations of the portal vein and vena cava to establish a collateral circulation about the liver (12). 3 to 5 weeks later the animals were fasted for 72 hours and given water which they drank freely. The long fasting period was found necessary, for the respiratory quotient of a previously well nourished rabbit, which is fasted, does not fall in less than 3 days. At the end of this time if a preliminary respiratory quotient determination was found to be low, as it usually was, blood specimens were taken for sugar estimations and hepatectomy done under ether anesthesia.

At once after operation blood sugar determinations were again made and a continuous intravenous injection, of isotonic (5.4 per cent) glucose solution, begun. The change in the blood sugar percentage found at this second estimation deter-



TEXT-FIG. 1. The Blood Sugar Concentration of Rabbits Deprived of the Entire Liver and Given Small Amounts of Glucose during the Interval between the Preoperative and Postoperative Respiratory Quotient Determinations.

The blocked in areas indicate the period during which respiratory quotient determinations were made and their height above the base line the blood sugar levels. Blood sugar estimations were done after each of the preoperative respiratory quotient determinations and their level plotted accordingly. In each instance a blood sugar estimation was made again immediately after the operation for hepatectomy. The changes in concentration are shown by the dotted lines. At various intervals thereafter the blood sugar concentration was determined in each animal and the variations depicted by the continuous lines. As the blood sugar levels were known both before and after the postoperative respiratory quotient determinations were made, the chart shows the approximate level of blood sugar concentration during those periods. It is obvious that the blood sugar concentration fell during the postoperative respiratory quotient determinations.

mined how much glucose was later given, the amount being that deemed necessary just to maintain the blood sugar concentration above the level of 100 mg. per cent. Experience soon taught us that it was safer to give too much glucose than

too little, for in two experiments the animals entered the state of hypoglycemic collapse while in the respiration chamber before the respiratory quotient determinations could be made. These experiments, of course, were ruled out. Three of the instances plotted in Text-fig. 1 received slightly too much glucose and a distinct rise in the blood sugar level followed. At various intervals blood sugar determinations were made as guides to the rate at which glucose was injected later.

After the operation, during the continuous glucose injection, the animals sat quietly on a warmed pad, with only occasional shifting movements.

About 5 to 6 hours after removal of the liver a second estimation of the respiratory quotient was begun; and in one instance a third was made 24 hours after hepatectomy. Ordinarily the animals remained in the respiratory quotient chamber about $1\frac{1}{2}$ hours. The glucose injection was continued during this period, the fluid entering the chamber through a glass tube connected in turn with fine rubber tubing about 8 inches long, to which the needle was attached. The needle inserted in the ear vein was kept in place by two weak bulldog clips and the rubber tube supported along the animal's back by an adhesive strip to prevent its occlusion by a kink should the rabbit shift its position. In all of the experiments the glucose injection was successfully carried out.

To determine changes in the blood CO_2 and sugar, specimens of venous blood were taken in every instance, immediately before and again after the animal's sojourn in the respiration chamber. To obtain blood samples in these experiments cardiopuncture was not necessary since the animals presented greatly enlarged collateral abdominal veins from which blood was taken, under oil, in paraffined syringes.

As in the previous experiments, we shall consider only the cases in which the metabolic rate remained high after operation and no significant blood CO_2 changes could be demonstrated.

Findings.

It was deemed sufficient to carry on experiments with hepatectomized rabbits until 6 instances had been obtained which fulfilled the conditions mentioned above. Table III gives a survey of the findings with the 6 different animals, on one of which (No. 4) a third respiratory quotient was obtained 24 hours after operation. In Text-fig. 1, we have charted the blood sugar curves of these animals in such a way as to show the *probable* blood sugar level during the period of respiratory quotient determinations.

Of the 6 instances, 4 showed practically no change in the respiratory quotient after liver removal, although in 1 of these (No. 4) a late determination was made as long as 24 hours after the operation. In

TABLE III.

The Respiratory Quotient and Metabolic Rate in Rabbits Deprived of the Entire Liver.*

The respiratory quotients of 6 fasted rabbits before and after removal of the liver are shown, together with the rate of oxygen consumption during each respiratory quotient determination. After hepatectomy all these animals received glucose. In Columns 4 and 9 the "noncarbohydrate" respiratory quotient has been calculated as explained in the text.

No.	Preoperative		Postoperative							
	Preliminary respiratory quotient	O ₂ consumption	Second respiratory quotient	"Noncarbohydrate" respiratory quotient	Hours after operation	O ₂ consumption	Third respiratory quotient	"Noncarbohydrate" respiratory quotient	Hours after operation	O ₂ consumption
	A	B	C	D	E	F	G	H	I	J
		mg per min				mg per min				mg per min
1	0.751	17.22	0.757	0.715	6	16.40	0.862†	—	8†	15.91
2	0.772	20.53	0.766	0.749†	5½	19.35	—	—	—	—
3	0.779	18.03	0.767	0.709	5½	17.08	—	—	—	—
4	0.783	19.84	0.794	0.729	5½	20.18	0.786	0.715	24	18.84
5	0.765	17.82	0.833§	0.765	5	15.48	—	—	—	—
6	0.786	21.12	0.881§	0.748	5½	16.92	—	—	—	—

* Expressed in terms of O₂ consumption per minute.

† Excess of glucose given as described in text.

‡ Did not receive enough glucose during respiratory quotient determination.

§ Received too much glucose before and during respiratory quotient determination. Blood sugar high.

TABLE IV.

Blood CO₂ of Rabbits Deprived of Total Liver Immediately before and after the Period of Respiratory Quotient Determination.

No.	Before entering respiration chamber	After entering respiration chamber	Hours after operation
	vol per cent	vol per cent	
1	43.35	43.14	6
2	39.35	38.98	5½
3	40.18	39.95	5½
4	41.63	41.34	5½
4	44.80	45.40	24
5	37.16	37.58	5
6	45.60	45.42	5½

2 instances an appreciable rise in the respiratory quotient occurred. It may here be remarked again that of necessity all the animals received continuous intravenous glucose injections during the interval between the operation and the completion of the respiratory quotient estimation.

In both of the animals in which a rise in the respiratory quotient occurred, Nos. 5 and 6, a marked fall in blood sugar was noticed immediately after the liver ablation (see Text-fig. 1). To prevent the development of hypoglycemic symptoms, much more glucose was given to these animals than to the others. In Rabbit 6, far too much glucose was given, 200 mg. per kilo per hour, and the blood sugar level stood at 159 mg. per cent at the beginning of the respiratory quotient determination. The other instance showing a rise in the respiratory quotient (No. 5) also received much more glucose per kilo per hour than those in which no rise was found. As Text-fig. 1 shows, the blood sugar concentration in this instance was abnormally low after operation. The animal was consequently given 185 mg. of glucose per kilo per hour instead of the usual 100 to 130 mg. Although the blood sugar concentration in the animal was not high immediately before the period of respiratory quotient estimation, it is to be noted that the animal had received this excess sugar.

The findings show beyond doubt that fat combustion continues actively in the animal without a liver.

The Effect of Increased Sugar Administration on the Respiratory Quotient of Hepatectomized Rabbits.

During the respiratory quotient determinations, the period that is to say when the blood sugar level could not be ascertained, the amount of glucose given was arbitrarily cut down to avoid the production of a sugar combustion sufficient to mask any existing one of fat. On the assumption that this glucose, given during the periods of respiratory quotient estimation, was burned we have calculated the hypothetical "non-carbohydrate-burning respiratory quotients," shown in Columns D and H of Table III. These approach closely in some cases the figure 0.71, that of a wholly fat burning respiratory quotient. As Text-fig. 1 shows, the blood sugar concentration of each animal was lower at the end of the respiratory quotient determination than before,

a finding which would support the assumption that the sugar introduced had been used up.

In calculating the hypothetical "non-carbohydrate-burning respiratory quotient" of Columns D and H in Table III we are for the moment assuming that the administration of glucose raised the respiratory quotient. To test the point one experiment was done.

In the experiment on Rabbit 1 a respiratory quotient of 0.757 was obtained between the 6th and 7th hours after operation. For 6 hours prior to this the animal had been given 100 mg. of glucose per kilo per hour by continuous intravenous injection. The blood sugar level fell from 126 mg. per cent 1 hour after liver removal to 0.098 just before the respiratory quotient determination. During the period of the respiratory quotient estimation the glucose dosage was further decreased to 80 mg. per kilo per hour, causing a further fall in the blood sugar level to 0.086, as Text-fig. 1 shows.

The animal was then given an intravenous injection of 5 cc. 5.4 per cent glucose and the rate of the continuous injection increased to the level of 300 mg. per kilo per hour. 20 minutes later another respiratory quotient determination was begun. This required 1½ hours, during which glucose was given at the rate of 300 mg. per kilo per hour. The quotient rose to 0.862. To avoid confusion with the findings in the other instance the latter part of the experiment has been omitted from Text-fig. 1.

DISCUSSION.

It is not to be inferred from our findings that the respiratory quotient must be low under all conditions after removal of the entire liver or a 90 per cent fraction of it. In this paper we wish to emphasize merely that the organism deprived of the liver is still capable of burning fat adequately for its needs. An existing fat combustion may be easily masked, as shown by our experiments in which too much glucose was administered, purposely or inadvertently to the liverless animals. And the respiratory quotient may be high in a variety of circumstances after hepatectomy. Thus, for example, if it is high before operation, it will tend to remain so thereafter. One of our "unsuccessful" experiments serves to illustrate this point.

Ablations of 90 per cent of the liver were carried out on two rabbits which had been fasted but 36 hours. This was done following the preliminary respiratory quotient estimations but before the results had been calculated. At operation the stomachs of the animals were found moderately distended with food. The preliminary respiratory quotients were 0.865 and 0.851, respectively. In both

instances, the respiratory quotient was found high 6 to 8 hours after operation, 0.811 and 0.826, respectively. Autopsy disclosed quantities of undigested food in the stomachs, sufficient reason for the high quotients. In another experiment, a freshly fed rabbit on a carbohydrate diet was subjected to the same procedure. The respiratory quotient which was close to unity (0.951) remained at this figure 6 hours later.

In another rabbit with a preliminary respiratory quotient of 0.731 the second determination was purposely taken 2 hours after operation and found to be 0.840. Guided by this finding we considered it advisable as routine to wait several hours after hepatectomy before attempting the second respiratory quotient estimations.

From the evidence given here one can only say with certainty that fat combustion continues unaffected after removal of the liver of animals already burning fat before operation. The state in which they are burning fat can be brought about by fasting, for about 3 days, animals previously well fed. The freshly fed rabbit on a carbohydrate diet shows as a rule a high respiratory quotient, and so does one fasted for less than 48 hours. On the other hand the fasting period should not be unduly prolonged. For one would of course expect a respiratory quotient indicative of protein combustion in an animal suffering from severe inanition.

Some experimenters have in the past employed the respiratory quotient as an indicator of the type of metabolism existing in animals after attempted exclusion of the liver from the general circulation by the ligation of vessels. In 1910 Porges (18) and Porges and Solomon (19) found a rise in the respiratory quotient of rabbits and depancreatized dogs following ligation of the abdominal aorta, the inferior vena cava, the portal vein and the hepatic veins. They concluded that the oxidation of fat and protein proceeds within the body only in the presence of the liver. In these experiments the animals lived but a few hours, rapidly becoming moribund. Rolly (20), too, reported changes in the respiratory quotient after interferences with the circulation of the liver but found them inconstant. Verzar (21, 22) obtained inconstant results after partial exclusion of the liver by anastomosis of the portal vein with the inferior vena cava. Grafe and Fischler (23) reported no change in the respiratory quotient of dogs with Eck fistulas. Fischler and Grafe (24) ligated the hepatic artery in Eck fistula dogs and observed usually a rise in the respiratory quotient within 6 hours of the operation. But it is to be noted that the respiratory quotient returned to the preoperative figure in the animals surviving more than 6 hours. Bohm (25) excluded the abdominal organs in depancreatized dogs and found but little rise in the respiratory quotient. Still later Grafe and Denecke (26) extirpated the liver in dogs several weeks after an Eck fistula had been formed and found the respiratory quotient low—in one instance 0.774 after operation.

Several explanations for these varying results have since come to hand. As is now well known, from the work of Mann (27) and others, an evident liver lack can only be brought about by almost complete removal or destruction of the organ. Rich (28) has recently demonstrated the futility of attempted exclusion of the liver from the circulation through the methods practiced by most of the earlier workers. Murlin, Edelmann and Kramer (29) have pointed out that radical ligations, excluding as they do much blood and tissue, should be carefully controlled. These authors have demonstrated that changes in the relationships of the blood gases follow sudden clamping of the abdominal aorta which are of themselves sufficient to account for the results of the earlier workers without the need to invoke, as explanation, a change in the metabolism of the animals.

Although no radical circulatory interference, such as complicated these various findings, has been brought about in our experiments we have thought best to rule out the possibility of errors due to changes in the blood CO_2 and have done so by considering only instances in which no variations of the sort occurred.

Mann (27, 30) has reported a series of carefully conducted studies upon the respiratory quotient of hepatectomized dogs. In his experiments the respiratory quotient tended to approach unity after removal of the liver. That the quotient in dogs need not necessarily be close to unity after hepatectomy is shown by the work of Markowitz (31).² From our findings on rabbits and the figures given in his paper we believe that the respiratory quotient of hepatectomized dogs would be found low 6 hours after the operation if fat combustion were duly established before liver removal and the giving of glucose afterwards reduced to a minimum.

SUMMARY.

Fat combustion is carried on adequately in rabbits deprived of the liver or brought into a condition of extreme liver insufficiency. Even 24 hours after hepatectomy fat combustion goes on as well as in the normal animal. Evidently the liver plays no essential part in the breaking down of fat.

² Following the completion of this work, F. C. Mann and W. M. Boothby reported experiments in the *American Journal of Physiology*, 1928, lxxvii, 486, which show an increase in the respiratory quotient of dogs immediately after removal of the liver. A subsequent return of the respiratory quotient 17 hours later to a figure but slightly above the preoperative one was also observed.

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ON THE MECHANISM OF OPSONIN AND BACTERIOTROPIN ACTION.

I. CORRELATION BETWEEN CHANGES IN BACTERIAL SURFACE PROPERTIES AND IN PHAGOCYTOSIS CAUSED BY SERA OF ANIMALS UNDER IMMUNIZATION.

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The opposing views of the early cellular and humoral schools of immunologists were to some degree brought into harmony by the demonstration by Denys and Leclef (1), Wright and Douglas (2) and others that phagocytosis could be greatly increased by the presence of serum. Components of normal sera promoting phagocytosis only in relatively high concentration were called by Wright and Douglas *opsonins*. The specific components of immune serum, active either in high or low concentration, have been termed *bacteriotropins* (3). It was shown that the action of opsonins and bacteriotropins was chiefly upon the bacteria or other cells phagocytized, not upon the leucocytes. Treatment by appropriate sera prepared bacteria or other cells for phagocytosis even after the removal of the serum.

Study of the phagocytosis of particulate matter brought out the fact that a number of factors in the phagocytic system could influence the degree of phagocytosis. Thus phagocytosis of carbon particles suspended in sodium chloride solution could be increased somewhat by addition of small amounts of calcium chloride, ethyl alcohol or iodoform, and decreased by departure of the suspending sodium chloride solution from isotonicity (4). Manganese dioxide particles showed an attraction for leucocytes and were very rapidly taken up by them; no such attraction could be demonstrated for carbon or quartz particles (5). Effects on the activities of delicate living cells like leucocytes by factors in their environment are not surprising. However, such effects are not comparable to the enormous increases in phagocytosis produced by the action of bacteriotropins on the cells to be phagocytized.

Important progress was made by formulation of the interfacial tension relations in phagocytosis through the work of Rhumbler (6), Tait (7), and especially of Fenn (8). Fenn showed that in a given phagocytic system the amount of phagocytosis was proportional to the number of collisions between leucocytes and

particles (9). The chance that any given collision may result in phagocytosis would seem to depend to an important degree upon the *interfacial tension relations* at the colliding surfaces; these must be such that the leucocyte and particle will adhere and that the former will spread around and engulf the latter (8). It is understood, however, that the consistency of the protoplasm (10, 11, 12) of the leucocyte and possibly other factors may also enter into the phenomenon.

If then the surface properties of cells are of great importance in determining phagocytosis, have sera any such effects on cell surfaces as might account for their opsonic and bacteriotropic efficiency? Much evidence has been accumulating of late years to indicate that sera do profoundly change the surfaces of cells with which they interact. Studies on bacteria (13), erythrocytes (14), and spermatozoa (15), using the method of cataphoresis, and on erythrocytes (16) and acid-fast bacteria (17), using a method dependent on interfacial tension, have shown that all of these cells do, indeed, undergo certain characteristic changes in surface properties with serum sensitization. The present experiments indicate that these changes are intimately related to phagocytosis.

The experiments described in this paper and the one immediately following represent the first stage of a study directed toward analysis of the mechanism of opsonin and bacteriotropin action in physical-chemical terms. They are intended to answer the question, "What changes do sera effect in acid-fast bacteria in preparing them for phagocytosis?"

Methods.

Various strains of acid-fast bacteria, treated with serial dilutions of normal and immune sera, have been studied as follows:—(1) The bacteria remained in serum dilutions overnight and the *agglutination* readings were then made for each tube. (2) The serum-bacterial mixtures were strongly centrifugated and the sediments were *resuspended* by shaking until the untreated control tubes showed even suspension. (3) The sensitized bacteria were washed and their wetting or interfacial tension properties were then estimated in the *interface reaction*. The bacteria were observed microscopically in an oil-water boundary surface. (4) The cataphoretic velocity of the washed, sensitized bacteria was measured in a microcataphoresis cell. These 4 reactions together gave a picture of the cohesiveness, of the wetting properties, and of the surface potential difference of the bacteria tested for phagocytosis. (5) While such data were being obtained mixtures of rabbit leucocytes with, (a) bacteria and serum dilutions, or, (b)

sensitized, washed bacteria were rotated in stoppered vials on a Robertson agitator (18). Smears were made from each mixture, fixed and stained, and 100 (or 200) leucocytes in each smear were observed microscopically. The percentage of leucocytes which had taken up bacteria was recorded.

Such details of the several reactions as seem necessary are given below:

Bacteria were removed with a platinum loop from the surface of a glycerol-agar slant. They were rubbed against the bottom of a Pyrex test tube by a glass rod with rounded end. A few drops of 0.85 per cent sodium chloride solution were added after the first grinding, and the bacterial mass was rubbed up into a paste; this was diluted with 0.85 per cent sodium chloride with further grinding and shaking. This suspension was largely freed from clumps by centrifugation and brought to the desired turbidity with saline.

1. *Agglutination*.—1 cc. of the bacterial suspension was mixed with 1 cc. of each serum dilution. The tubes were left in the ice-box over night and in the morning were usually removed to the 37° room for 30 to 60 minutes before reading. Agglutination or sedimentation was read without shaking up the sediment. ++++ indicates complete agglutination with clearing, +++, ++, + and tr. indicate decreasing degrees of agglutination or sedimentation. The lesser degrees of agglutination recorded in many experiments with mammalian tubercle bacilli may well have indicated only sedimentation and not macroscopic agglutination, strictly speaking. Such sedimentation was never considered positive however, unless distinctly in excess of the sedimentation in the saline control tube.

2. *Resuspension*.—Under various special conditions, for instance with mammalian tubercle bacilli, agglutination as ordinarily carried out is unsatisfactory. To meet these cases a modified agglutination or "resuspension" reaction has been developed (19). After making the agglutination readings, all tubes were centrifugated at high speed until the bacteria were completely sedimented or practically so. The supernatant fluid was decanted and two drops of 0.85 per cent sodium chloride solution were added to the bacterial sediment in each test tube. The tubes were arranged in a rack with the control tubes in the middle, *i.e.*, tubes in which the bacteria had been mixed with 0.85 per cent sodium chloride solution without serum. The rack was now shaken uniformly until the sediment in the control tubes was first brought into even suspension. The bacteria which had been sensitized resuspended in flocculi whose coarseness increased with the concentration of serum and with the affinity of the serum components for the particular bacteria used.

This reaction has the advantage of eliminating certain imperfectly controlled variables in the second or flocculation stage of the agglutination reaction. The bacteria after sensitization are forcibly pressed together by centrifugal force.

Their subsequent resuspension depends primarily upon and gives a roughly quantitative estimate of their cohesion.

3. *Interface Reaction*.—After resuspension, 0.85 per cent sodium chloride solution was poured into each tube, all tubes were again centrifugated, the supernatant liquid was decanted, and the sediments were again resuspended. A portion of this sensitized washed suspension in each tube was used for the interface reaction (17) and a portion for cataphoresis. In the present experiments, the bacteria were observed microscopically in the interface between tricaprillin and 0.85 per cent sodium chloride solution. The degrees of alteration of the wetting properties of the bacteria after sensitization are charted as plus signs.

4. *Cataphoresis*.—A slightly modified form of the Northrop-Kunitz (20) microcataphoresis cell was used. For details see (21). The cataphoresis cell was mounted over a dark-field condensor. Three readings were made at each of 3 levels, namely, at $\frac{1}{12}$, $\frac{1}{6}$, and $\frac{1}{3}$ of the distance from the bottom to the top of the inside of the cell. A better technique used in the later experiments was to make 3 readings at each of the 2 "stationary levels," namely, at $\frac{1}{10}$ and at $\frac{9}{10}$ of the inside depth of the cell (22). The algebraic sum of the rates of migration at the several levels was used for calculation. The cataphoretic velocity is given in μ per second per volt per centimeter fall in potential along the cataphoresis cell. If we make certain assumptions (23), which are not rigidly accurate, however, velocities may be converted into millivolts potential difference at the bacterial surface by multiplication by 12.6.

Phagocytosis.—The importance of using serial dilutions of sera in careful phagocytosis work has long since been demonstrated (3, 24), but has been neglected. Moreover much work in phagocytosis has been vitiated and discredited by the difficulties and uncertainties of the Leishman-Wright opsonic index method. Introduction of the beautiful technique of Robertson and his co-workers (18) has therefore marked an important advance. The Robertson apparatus has been adapted to the needs of the present experiments, and a technique for staining and handling several titration series at the same time has been developed.

Leucocytes.—Exudative leucocytes from rabbits were used as the phagocytic cells according to a method elaborated in Hamburger's laboratory (25). They were obtained by injecting about 200 cc. of sterile 0.9 per cent sodium chloride intraperitoneally (on the right side about the mid-clavicular line and midway between costal margin and pelvis) into a rabbit by means of a hollow needle attached to a rubber tube and funnel; after 3 to 4 hours about 80 to 100 cc. of the fluid was recovered by puncturing the peritoneal cavity with a stout (No. 15 bore) needle near the mouth of which a few side openings had been drilled. The fluid was received in a flask containing a solution of 0.7 per cent sodium chloride + 1.1 per cent sodium citrate in proportion of 3 parts of peritoneal fluid to 1 part of saline-citrate solution. (In order to obtain fluid very rich in leucocytes an injection of 200 cc. of sterile 0.9 per cent sodium chloride solution was given on the evening before the experiment, to be followed in the morning by a second injection as described above.) The suspension was centrifuged for 2 minutes at a speed

of 1100 revolutions per minute; the centrifuge was started and stopped gradually; the supernatant fluid was poured off and the sediment resuspended in 0.9 per cent sodium chloride and centrifuged again for 2 minutes at the same speed. About 0.1 cc. of leucocytes were obtained from 15 cc. of peritoneal fluid. Usually the leucocytes from 3 rabbits were used, furnishing about 10 cc. of a very dense leucocytic suspension. Approximately 95 per cent of the cells were polymorphonuclear leucocytes; disintegrated cells and large monocytes constituted the remaining 5 per cent. The rabbits showed no ill-effects from the injection and withdrawal of fluid. The same rabbits were used weekly for 8 months. When leucocytes were obtained oftener a different series of rabbits was employed.

Phagocytic Mixture.—Into a series of small hard-glass vials of uniform size (50 mm. in length, 9 mm. inside diameter), 0.1 cc. of serum dilutions was placed (a fresh pipette was used for each dilution to insure greater accuracy). Immediately before adding 0.2 cc. of the leucocytic suspension (which was never more than $\frac{1}{2}$ hour old), 0.1 cc. of bacterial suspension was introduced. Each of the vials contained, therefore, 0.4 cc. of fluid. They were stoppered with freshly paraffined well-fitting corks, and immediately placed on a Robertson rotating machine. The speed of rotation was 4 times per minute; the rotation was continued for 15 minutes. Temperature was that of the room, $25^{\circ} \pm 1.5^{\circ}\text{C}$. At the end of this period the tubes were quickly placed in racks and plunged in ice water for several minutes. A minute drop of human serum (heated for 30 minutes to 56°) was added to each vial immediately before making the spreads; this caused the leucocytes to adhere to the slide. The first vial of each series did not require the addition of serum.

Making of Spreads.—Spreads were made on thoroughly cleaned and polished numbered glass-slides. A small drop of the mixture was placed near one end of the slide with a clean pipette, using a fresh pipette for each vial, and drawn across the slide by a spreader (a slide with the corners broken off); the edge of the spreader was then dipped into a beaker of water and wiped clean and dry. The spread slide was rapidly dried by holding it before an electric fan which was standing in front of a radiator or electric heater. Rapid drying is essential for obtaining well-spread non-contracted leucocytes. The dried slides were exposed for 2 to 10 minutes to formalin vapors (by placing them, spread side downwards) over flat dishes containing undiluted formalin. After removal from formalin vapors they were placed in a row and the flame of a Bunsen burner passed rapidly over them.

Staining.—The fixed spreads were stood in staining racks and placed for 10 minutes in a covered jar containing Ziehl-Neelsen carbol-fuchsin previously heated to 70°C . They were then thoroughly rinsed in water. Each slide was decolorized separately by shaking it, with forceps, for 5 to 10 seconds in 10 per cent sulfuric acid. (This was found to decolorize the cells satisfactorily; the bacilli were brilliantly stained.) The slides were counterstained, usually for 20 seconds, in a 1:10,000 Azure II solution; (the dilution was made from a more concentrated solution; the diluting distilled water was rendered slightly alkaline by adding 0.3

cc. of a 15 per cent sodium carbonate solution to each 200 cc. of water). The staining solution was always freshly prepared. The slides were then rapidly washed, and dried, first between blotting papers and then by holding them in front of an electric fan (or placing them in the incubator).

*Estimation of Degree of Phagocytosis.*¹—The numbers of leucocytes that had ingested bacilli were determined by counting 100 cells (sometimes 200 cells). Each of two observers counted 50 (or 100) cells; the results agreed very closely. Care was taken not to regard mere adherence to the leucocyte as evidence of phagocytosis. In the vast majority of instances there was no doubt as to whether a cell had ingested bacteria or not.

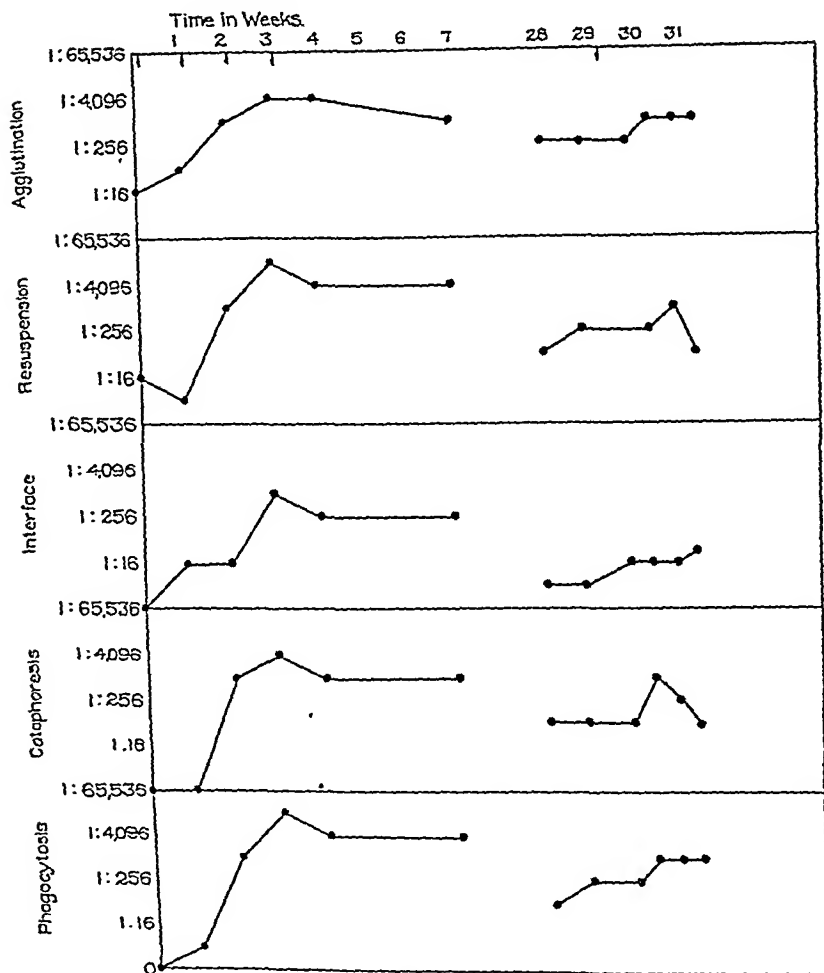
The agglutination, resuspension, interface and cataphoresis reactions were done at the Phipps Institute by one group of workers; portions of the sera and bacterial suspensions used in these tests were meanwhile sent to the other collaborators at the Medical School, by whom the phagocytosis tests were made. The results were not combined until both sets of observations had been completed.

By use of the reactions and procedure outlined above we are able to follow changes in the cohesiveness, the surface potential difference and the wetting properties of the bacterial surfaces, and to compare these changes with the increase in phagocytosis brought about by the same sera on other portions of the same bacterial suspensions. Positive resuspension and interface reactions each indicate increased cohesiveness between the bacteria of the suspension tested. A positive interface reaction indicates also bacterial surfaces less readily wet by oil than are those of the controls. The change (decrease) in surface potential difference is directly proportional to the decrease in cataphoretic velocity brought about by serum sensitization. Each of these changes in bacterial surface properties is closely correlated with the increase in phagocytosis brought about by serum, as will be shown in this and the two succeeding papers.

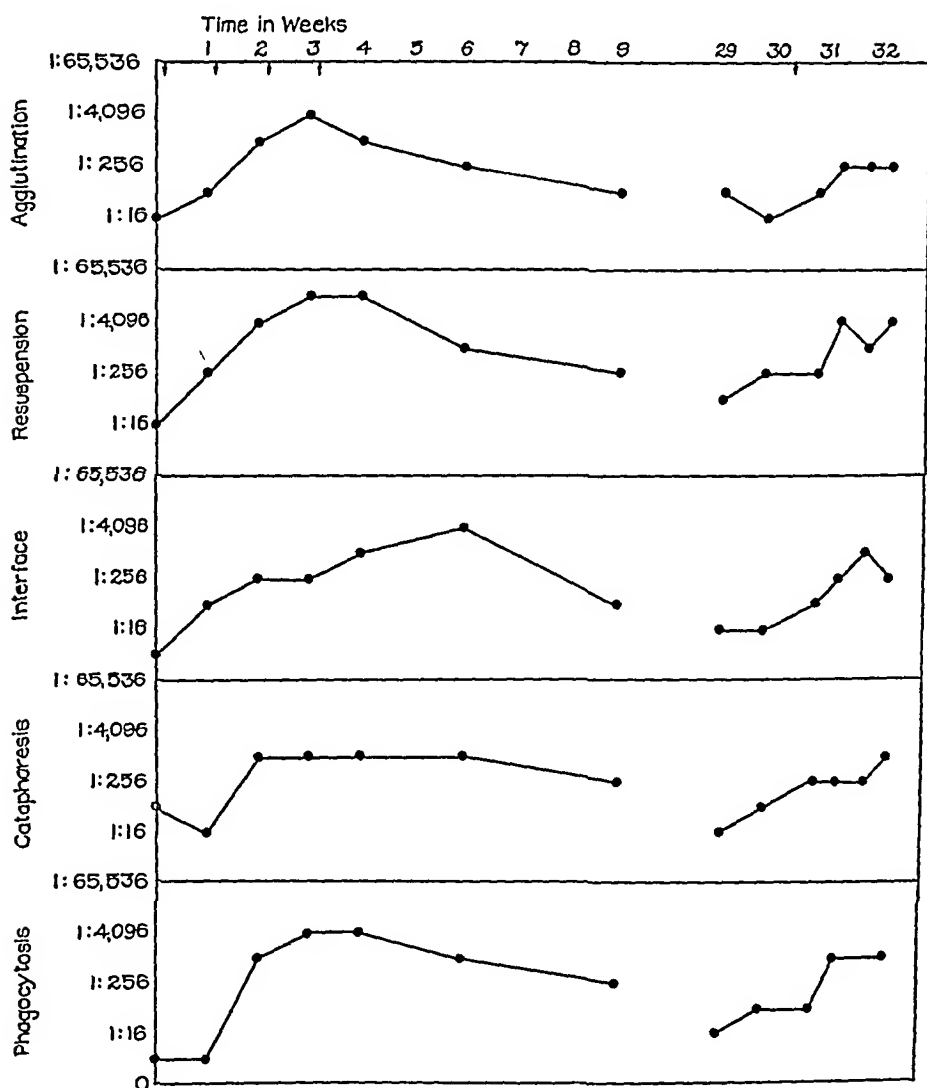
In Text-figs. 1 to 4 are plotted the titers for the several reactions of the sera of 4 rabbits, as withdrawn during the course of active immunization. The times in weeks between the beginning of the experiment and the bleedings of the rabbits are plotted on the abscissa axis.²

¹ In the present study phagocytosis is defined as ingestion of bacilli by polymorphonuclear leucocytes; the effect on the bacilli ingested is not here under consideration, nor is the question of phagocytosis by other types of cells than polymorphonuclear leucocytes.

² The sera were separated from the freshly clotted blood, and were inactivated for 30 minutes at 56°C. before use. The saline bacterial suspensions were ad-

Rabbit 19-72, injected with *M. chelonae*.

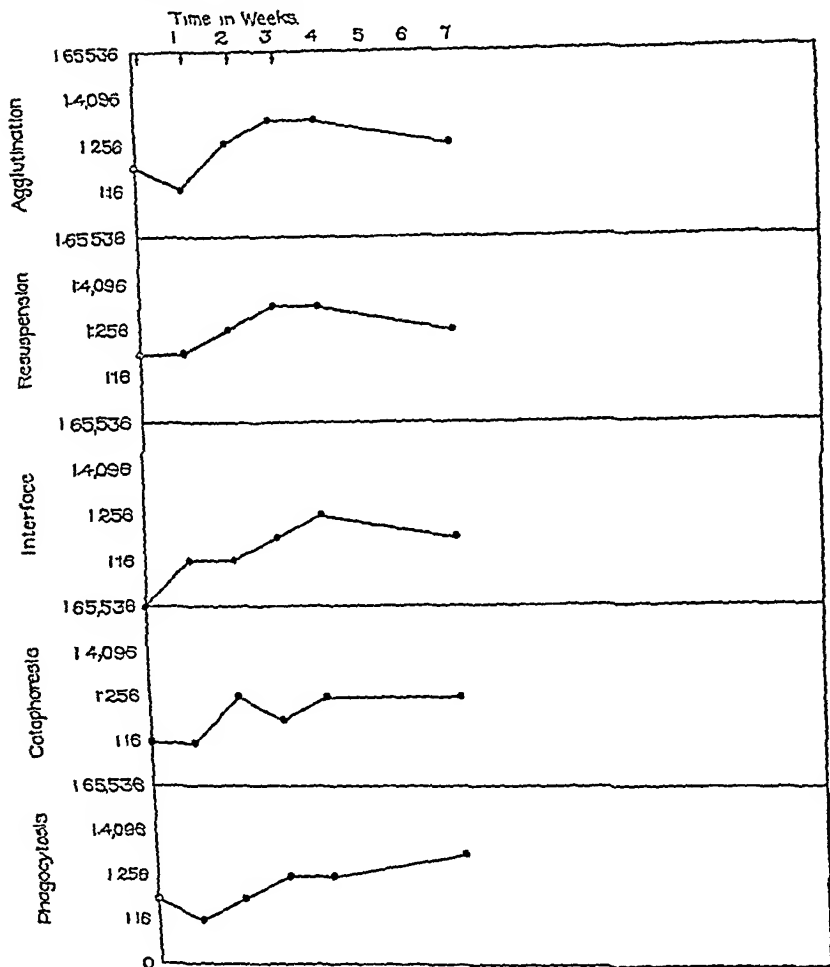
TEXT-FIG. 1. The course of active immunization of a rabbit with turtle bacillus. Titers of the several reactions are plotted against time in weeks. Time of intravenous injections of living antigen is indicated by arrows. Note the parallelism between phagocytosis and bacterial surface changes in this and succeeding text-figures.

Rabbit 20-02 injected with *M. avium* (Arloing strain).

TEXT-FIG. 2. Active immunization of a rabbit with an avian tubercle bacillus. The open circle (first cataphoresis titer) indicates titer not reached but estimated.

Intravenous injections of saline suspensions of the living bacilli used for immunization are indicated by small arrows on this axis. The

justed as nearly as possible by comparison with a turbidity standard to the same turbidity for each experiment; the living microorganism homologous with the antiserum was used for all tests.

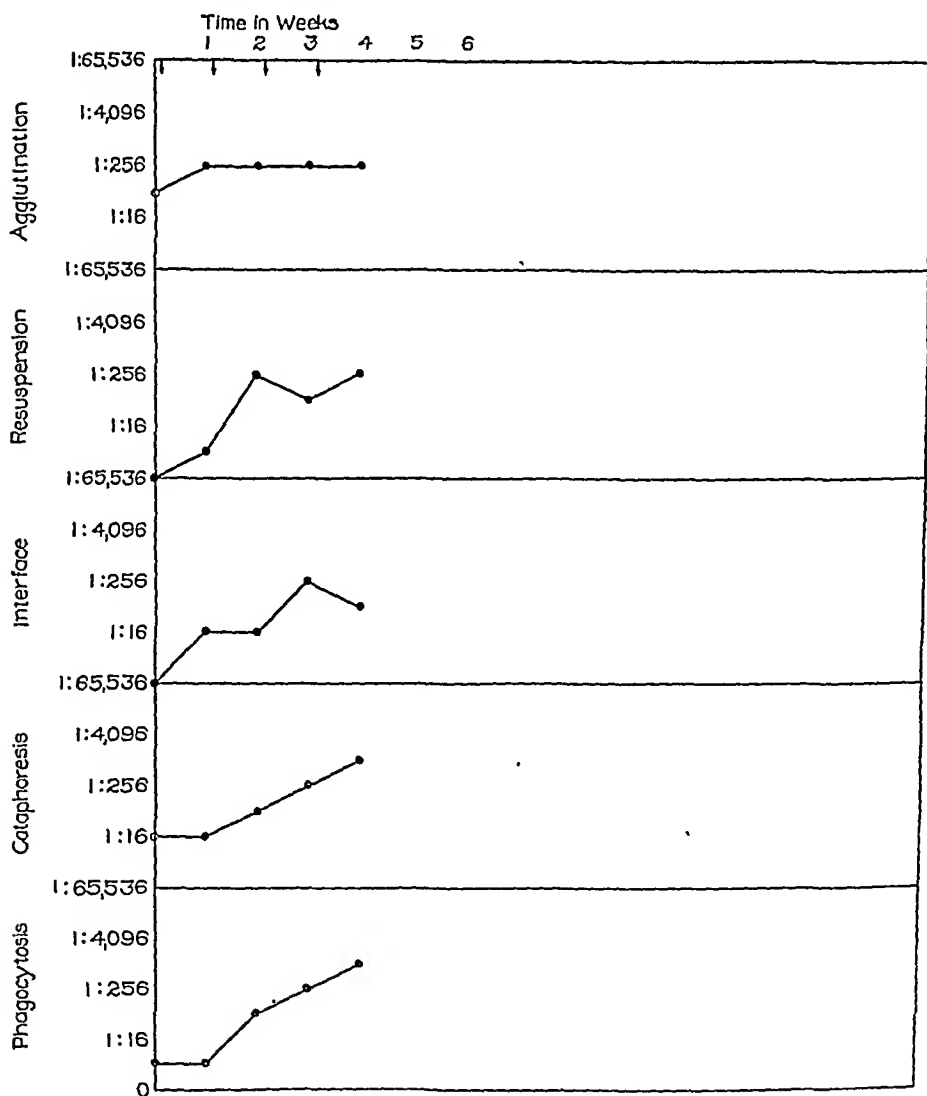
Rabbit 20-03, injected with *M. avium* (Prague strain).

TEXT-FIG. 3. Active immunization of a rabbit with an avian tubercle bacillus. Symbols as in Text-figs. 1 and 2.

titers of the sera are indicated on the ordinate axis; the highest dilutions of the sera giving clearly positive results are taken as titers; traces or doubtful reactions are not included.

Text-figs. 1 and 2 show the course of active immunization due to

Rabbit 20-37, injected with *M. tuberculosis* (Bovine III strain).



TEXT-FIG. 4. Active immunization of a rabbit with an avirulent bovine tubercle bacillus. Symbols as in Text-figs. 1 and 2.

4 weekly injections, the subsequent slight falling of titer, the titers after a 5 months interval and the rise following a final injection of the antigens. The rabbits yielding the data shown in Text-figs. 3 and 4 died of intercurrent disease after the first immunization.

The general correspondence is very striking between the bacterial surface changes and the increase in phagocytosis brought about by the sera at all the stages of immunization. The absolute titers for the several reactions do not necessarily correspond; this was to have been anticipated, however, since it had already been recorded, for instance, that the interface reaction was relatively insensitive (17), and that agglutination with mammalian tubercle bacilli (see Text-fig. 4) was unsatisfactory (19). An occasional point also is obviously off the correct curve (e.g., Text-fig. 2, interface value at 6 weeks). However the significant fact is the striking general parallelism of the titers indicating bacterial surface change and phagocytic increase; this parallelism is, we believe, complete, within the limits of experimental error.

Influence of the Intrinsic Properties of the Bacterial Surface.—Serum which prepares bacteria for phagocytosis, then, has in approximately corresponding degree altered the bacterial surface properties, i.e., increased the cohesiveness, decreased the surface potential difference and altered the wetting properties. The correspondence between these bacteriotropic and surface effects strongly suggests that they are due to a common cause, namely, to deposition of a sensitizing substance or substances from the serum on the bacterial surface. It has already been shown that serum agglutination involves a surface deposition of serum components (26).

It is of interest further to inquire: (a) whether phagocytosis and agglutination occur at a constant critical surface potential difference or whether a factor or factors other than potential difference enter into the determination of phagocytosis and agglutination, and (b) whether phagocytosis and agglutination can be formulated in terms of the properties of the sensitizing substance alone or are dependent both on the properties of the sensitizing substance and on the intrinsic properties of the bacteria undergoing sensitization. These questions may be answered from the data in Table I.

Table I gives the cataphoretic velocities at which phagocytosis and agglutination began, at which phagocytosis reached 50 per cent and at which agglutination approached completeness. Phagocytosis and agglutination are seen to begin and to reach the levels indicated within

TABLE I.

The Relation of Cataphoretic Velocity to Agglutination and Phagocytosis.

1. Antigen	2. Time of immunization	3. Unsensitized antigen	4. Phagocytosis begins	5. Agglutination begins	6. Phagocytosis 50 per cent or more	7. Agglutination +++ or +++++	8. Minimum potential difference
	weeks	c.v. in μ /sec.	c.v. in μ /sec.	c.v. in μ /sec.	c.v. in μ /sec.	c.v. in μ /sec.	c.v. in μ /sec.
<i>M. avium</i> (Arloing strain)	1	1.92	1.18	1.73	1.50	1.18	1.18
	2	1.67	1.50	1.50	1.34	1.30	0.92
	3	1.96	1.82	1.82	1.55	0.89	0.49
	4	1.83	1.71	1.55	1.00	0.61	0.43
	6	1.67	1.45	1.00	1.32	0.57	0.49
	9	1.93	1.64	1.32		0.75	0.63
<i>M. tuberculosis</i> (Bovine III strain)	1	1.37	0.92	1.34	Not reached	Not reached	0.92
	2	1.37	1.13	1.34	0.83	Not reached	0.83
	3	1.98	1.55	1.55	0.58	Not reached	0.58
	4	1.56	1.29	1.23	0.98	Not reached	0.54
<i>M. chelonae</i>	1	1.54	1.43	1.45	Not reached	1.43	1.43
	2	1.36	1.09	1.09	1.04	1.04	0.54
	3	1.24	1.17	0.95	0.95	0.65	0.59
	4	1.29	1.15	1.15	0.89	0.89	0.26
	7	1.78	1.59	1.41	1.20	1.20	0.41
							0.32
<i>M. avium</i> (Prague strain)	1	1.01	0.83	0.83	Not reached	Not reached	0.56
	2	1.05	0.75	0.82	0.60	0.60	0.60
	3	1.18	1.04	1.17	0.71	0.66	0.63
	4	1.10	0.78	1.22	0.57	0.52	0.52
	7	1.13	1.07	0.93	0.89	0.80	0.80

In columns 1 and 2 are given the antigen and the duration of immunization at the time the sensitizing serum was drawn; in column 3 the cataphoretic velocity (c.v.) of the control unsensitized antigen in salt solution is given.

In columns 4 and 5 are given the cataphoretic velocities corresponding to the beginning of phagocytosis and agglutination.

In columns 6 and 7 the cataphoretic velocities are given when phagocytosis has reached 50 per cent or over, and agglutination +++ to +++++.

The last column shows the lowest cataphoretic velocity reached by the antigen in each experiment.

The values in each category are averaged and the mean value is calculated as potential difference in millivolts.

The table shows that phagocytosis and agglutination occur within certain zones of cataphoretic velocity; but these zones are different for the different organisms.

fairly definite zones of surface potential difference,³ but the potential zones are different for the different organisms. Consider for instance the Arloing and Prague strains of avian tubercle bacilli of similar growth habit; the Arloing strain has a relatively high surface potential difference (average, 23 millivolts), and the Prague a low potential difference (average, 14 millivolts). The phagocytosis and agglutination titers for the Arloing strain fall at 20 and 19 millivolts and for the Prague strain at 11 and 12 millivolts; phagocytosis of Arloing reached 50 per cent at about 17 millivolts, of Prague at about 9 millivolts; agglutination approached completeness with Arloing at about 11 millivolts, with Prague at about 8 millivolts. Yet in the highest concentrations of immune sera both strains were reduced to about the same potential difference.

We conclude, therefore, (a) that phagocytosis and agglutination do tend to occur within certain zones of surface potential difference; since, however, these zones are different for the different organisms it is obvious that another factor or factors than potential difference must enter into these phenomena.

Further consideration of Table I and of other data leads to the conclusion (b) that phagocytosis and agglutination cannot be formulated in terms of the properties of the sensitizing substance alone but depend both on the properties of the sensitizing substance or substances and on the intrinsic properties of the bacteria undergoing sensitization.

Evidence pointing to this conclusion may be summarized as follows: Agglutination of Bovine III never approached completeness although the surface potential difference of this strain was reduced well below the level required for +++ or ++++ agglutination of Arloing or the turtle bacillus (*chelonae*). This difficult agglutinability is characteristic for mammalian tubercle bacilli in general, and has given rise to a number of artifices, e.g., agglutination at high temperature, agglutination of a substitute strain (Arloing strain), and the resuspension reaction (19). Phagocytosis of certain difficultly agglutinable strains of mammalian tubercle bacilli, on the other hand, may be high even without serum sensitization. This point is illustrated in Table

³ Surface potential difference is measured by and is proportional to cataphoretic velocity.

TABLE II.
Atypical Phagocytosis of Human Tubercle Bacillus Sensitized with Aged Serum.

<i>M. tuberculosis</i> , strain H 37, in anti H 37 serum	Serum dilutions							Sodium chloride control
	1:4	1:4 ²	1:4 ³	1:4 ⁴	1:4 ⁵	1:4 ⁶	1:4 ⁷	
Agglutination.....	++	+ to tr.	+	tr.	+ to tr.	tr.	tr.	tr.
*Resuspension.....	+++	+++	+++	+++ to +	+	++ to +	0	tr.
*Interface.....	More positive than control							
*Cataphoresis, μ /sec.....	0.70	1.16	1.87	2.12	2.14	2.14	2.28	2.31, 2.12
*Phagocytosis, per cent.....	5	17	88	81	85	89	88	71

* Bacteria sensitized and washed before testing.

II in which it is shown that a human tubercle bacillus of low virulence was phagocytized by 71 per cent of cells even without sensitization. Agglutination in the instance shown in Table II was slight although resuspension and cataphoresis showed the binding of agglutinin; the phagocytic prezone is characteristic of the aged serum used for sensitization; (see paper following). Tests with several other cultures of human tubercle bacilli gave 50 per cent or higher phagocytosis in the unsensitized 0.85 per cent sodium chloride suspension controls. Phagocytosis of this high order in the unsensitized controls has not been found with any type of acid-fast bacteria other than the human tubercle bacillus, and has not been found with all cultures of the human bacillus. It has already been recorded that the human tubercle bacilli we have studied in the interface differ from other types of acid-fast bacteria in their wetting properties (27).

Phagocytosis and agglutination are thus dependent both upon the properties of the sensitizing serum substance or substances and upon the intrinsic properties of the bacteria themselves.⁴ Serum sensitization tends to promote both phagocytosis and agglutination. The intrinsic properties of the bacterium may be either favorable or unfavorable to phagocytosis or agglutination and may even influence the 2 phenomena in opposite directions. Since agglutination is a matter of the cohesion of the bacteria themselves, and phagocytosis necessitates adhesion of the bacteria to, and engulfing by, leucocytes, the relative independence of the influence of the original intrinsic bacterial properties on the 2 phenomena is not surprising.

SUMMARY.

Methods are described for investigating the relation between phagocytosis of bacteria by polymorphonuclear leucocytes, and certain physical-chemical properties of the bacterial surface.

Serum sensitization causes the following changes in the properties of acid-fast bacteria: (a) increased cohesiveness, (b) decrease in

⁴ However it is possible that with maximum sensitization by a strong homologous serum the deposited sensitizing substance may form a complete film enveloping the sensitized bacterium. We are not sure whether the sensitizing film ever becomes complete or not. If it does, the original properties of the bacterial surface would doubtless no longer be factors in agglutination and phagocytosis.

surface electric potential difference, (c) decrease in wettability of the bacteria by oil, and (d) increased phagocytosis.

Tests have been conducted periodically with the sera of 4 rabbits under active immunization with as many strains of acid-fast bacteria; the parallelism between the alteration in bacterial surface properties and the promotion of phagocytosis by these sera has been, within the experimental error, complete.

The percentage of phagocytosis of a given bacterial suspension has been found to depend both upon the sensitizing serum component or components deposited upon the bacterium and upon the intrinsic properties of the unsensitized bacterial surface.

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ON THE MECHANISM OF OPSONIN AND BACTERIOTROPIN ACTION.

II. CORRELATION BETWEEN CHANGES IN BACTERIAL SURFACE PROPERTIES AND IN PHAGOCYTOSIS CAUSED BY NORMAL AND IMMUNE SERA.

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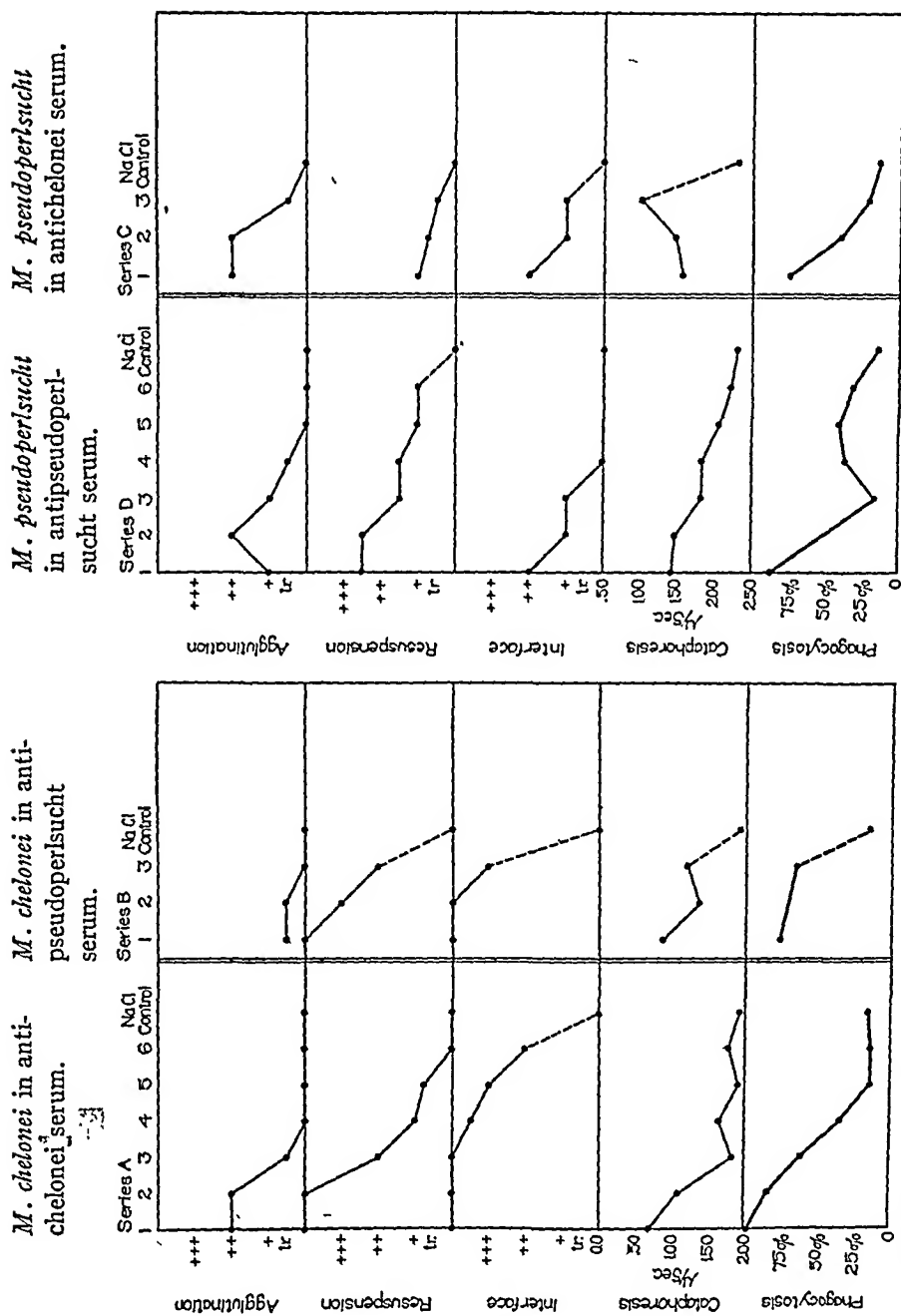
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(Received for publication, February 8, 1929.)

The sera of rabbits under immunization have been shown in the preceding paper (1) to cause parallel effects on the surface properties and the phagocytosis of acid-fast bacteria. Is this correspondence between the serum effects on surface properties and on phagocytosis a constant and necessary relation, or do circumstances exist in which the two effects are dissociated? It can be said at once that, in our experiments at least, whenever a serum has promoted phagocytosis it has invariably also altered the properties of the bacterial surfaces; and the surface and phagocytic effects have been in rough quantitative correspondence at least. Exceptionally circumstances have been found in which sera have altered bacterial surface properties, but have not increased phagocytosis. These failures of correspondence have all been with heated or aged sera or with sera of another species than the species from which the leukocytes were obtained.

Bacteriotropins.

Freshly drawn immune sera have regularly shown parallel bacteriotropic and bacterial-surface effects. An illustrative experiment is given in Text-fig. 1. The intensities of the several reactions are plotted as ordinates, and the final serum dilutions as abscissæ. It is seen that each serum when acting on its homologous strain caused increased cohesiveness (as indicated by the positive resuspension and interface



TEXT-FIG. 1. Homologous and cross reactions between turtle and pseudoperlsucht bacilli and their corresponding rabbit immune sera. Fresh sera heated to 56°C. for 30 minutes. Broken lines indicate that the titer was not reached. Numbers on horizontal axis indicate serum dilutions in powers of 4; thus 3 indicates a dilution of 1:4³ or 1:64. There is a general correspondence between bacterial surface changes and phagocytosis.

reactions), altered wetting properties (interface reaction), decreased surface potential difference (cataphoresis), and increased phagocytosis. The antipseudoperlsucht serum caused strong surface and bacteriotropic effects when used with the turtle bacillus (*M. chelonae*), and antichelonae serum gave moderate surface and bacteriotropic effects with the pseudoperlsucht bacillus.

The familiar fact that bacteriotropic action is exerted upon the bacterium not upon the leucocyte is demonstrated in Table I. In this experiment the bacteria were sensitized and then washed and suspended in saline solution. It is seen that phagocytosis is efficiently promoted by sensitization even in the absence of the sensitizing serum. The strains used were an avian and a bovine tubercle bacillus. Cross reactions are here almost as strong as those between homologous antigen and antibody; and again bacterial surface changes and increase in phagocytosis are in close correspondence.¹

Aged Immune Sera.—Rabbit immune sera which had been kept in the ice-box without preservative for about 16 to 18 months were kindly put at our disposal by Dr. J. D. Aronson. These had been kept in large test tubes stoppered with cotton; accordingly they had undergone some evaporation. Moreover a heavy greyish precipitate had formed in the bottom of each, and most of the sera showed some opalescence which could not be cleared by centrifugation. Typical effects produced by such sera are shown in Text-figs. 2 and 3.

Prague bacillus treated with homologous antiserum exhibits characteristic surface changes (Series A), (Text-fig. 2). Phagocytosis is also increased but only in high dilutions of serum, reaching its maximum at approximately 1:1000 and 1:4000 dilution. This surprising phagocytic prezone cannot be explained satisfactorily by postulating injury of the leucocytes by the aged serum, as is shown by Series C, in which the same anti-Prague serum caused a moderate increase in phagocytosis without prezone, with milk bacillus, in correspondence with moderate bacterial surface changes.² The anti-milk-bacillus serum with homologous antigen caused strong bacterial surface

¹ A graph of this experiment has appeared in Colloid symposium monographs (2).

² The tubes of Series C were contaminated with a coccus. This may have been responsible for the prezone in the resuspension reaction, which is not a usual finding.

Homologous and Cross Reactions between *M. avium* (Prague Strain) and *M. tuberculosis* (Bovine III Strain) and Their Corresponding Rabbit Immune Sera. Serum Dilutions Are Given as Powers of 4.

TABLE I.

In this experiment sensitized bacteria were washed before phagocytosis. It is seen that even in the absence of free serum there was a high degree of phagocytosis.

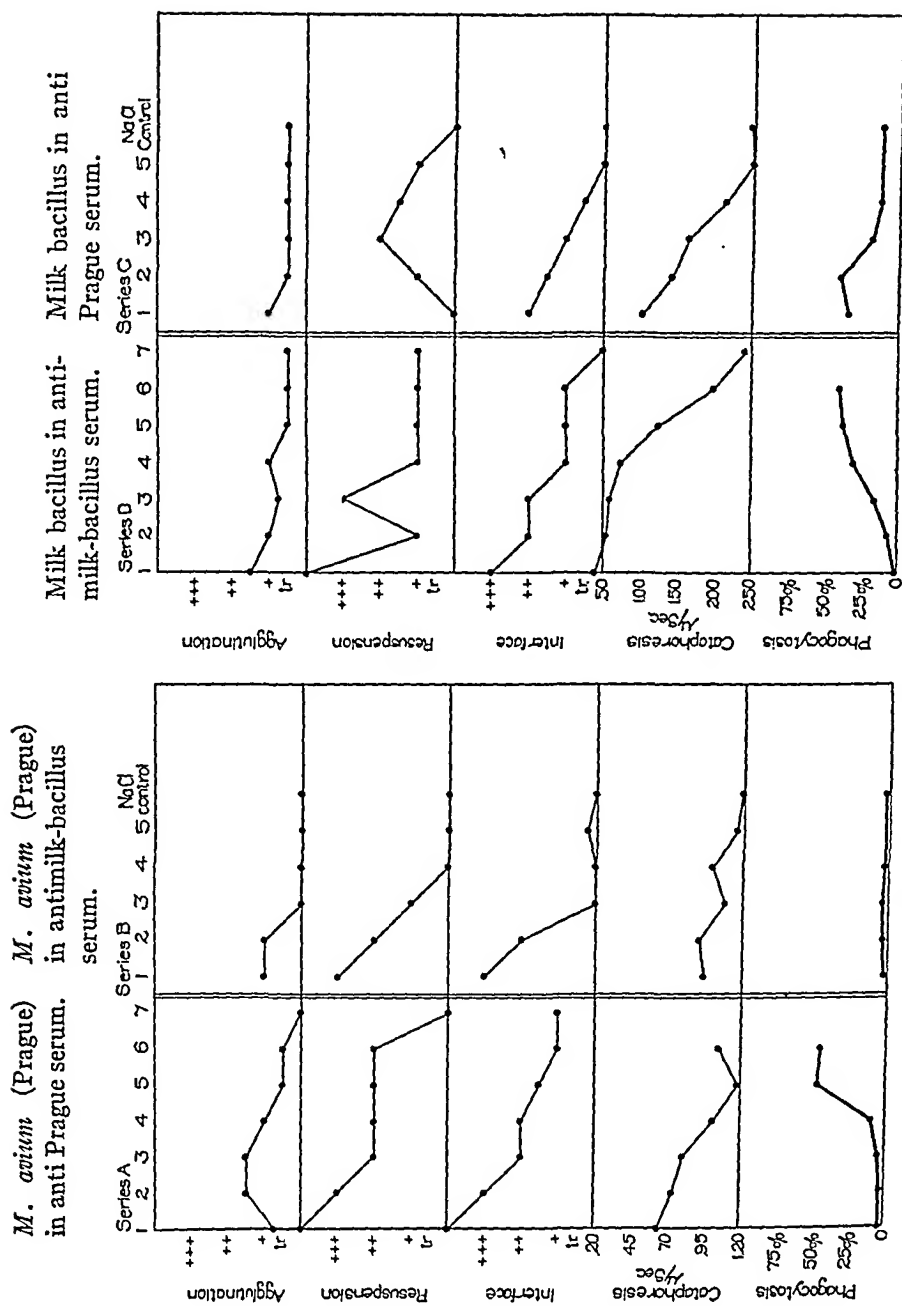
Serum dilutions									Sodium chloride control
1:4	1:16	1:64	1:256	1:1024	1:4096	1:16,384	1:65,536		
Series A. Prague in Anti-Prague Serum.									
Agglutination.....	++++ to +++	++++ to +++	++++ to +++	+	sl. tr.	sl. tr.	sl. tr.	sl. tr.	
Resuspension.....	++++	++++	++++	++	0	0	0	0	
Interface.....	++++	++++	++ to +	+	tr.	—	—	0	
Cataphoresis, μ /sec.....	0.34	0.21	0.45	0.70	0.83	0.86	0.93	1.02	
Phagocytosis, per cent.....	92	97	99	94	52	7	7	3	
Series B. Prague in Anti-Bovine III Serum.									
Agglutination.....	++++ to +++	++++ to +++	+++	+	+	tr.	—	sl. tr.	
Resuspension.....	++++ to ++	++++ to +++	+++ to ++	++ to +	0	0	—	0	
Interface.....	+++	+	tr.	0	—	—	—	0	
Cataphoresis, μ /sec.....	0.24	0.39	0.65	0.79	0.89	0.91	—	0.89, 0.98	
Phagocytosis, per cent.....	73	64	79	72	44	20	—	4	

Series C. Bovine III in Anti-Prague Serum.

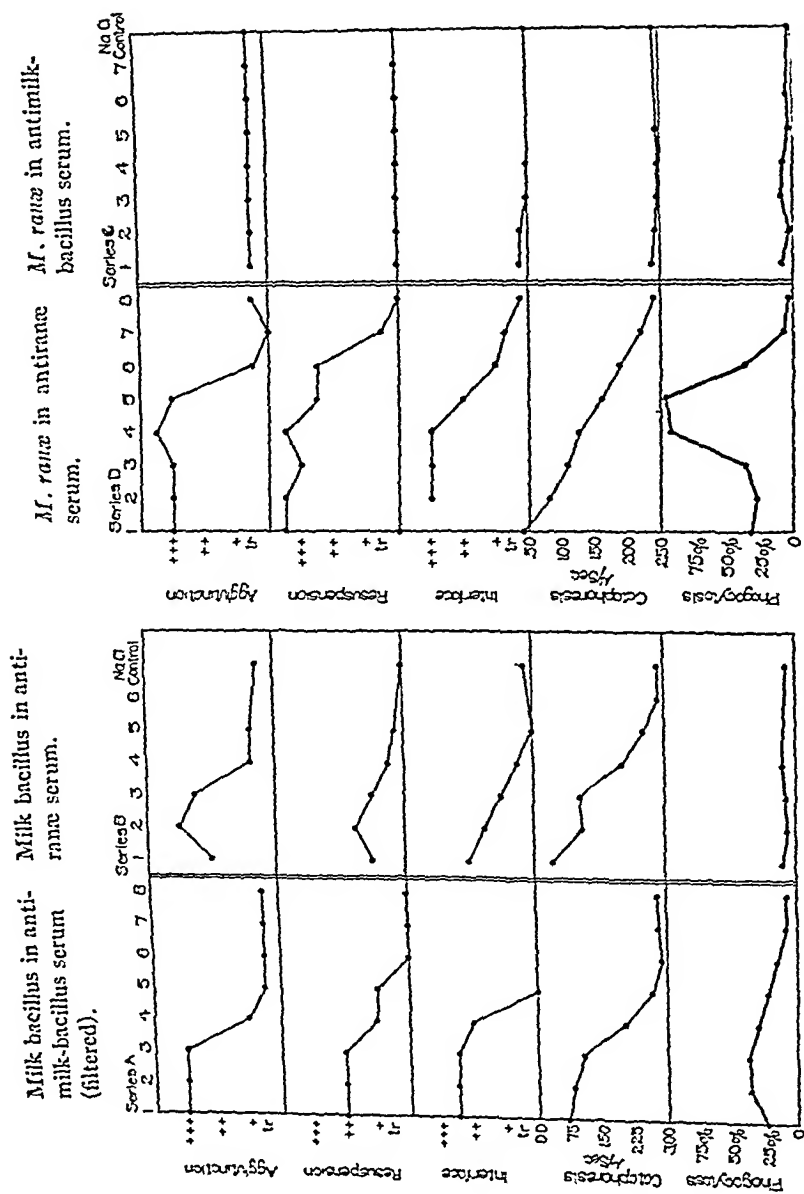
Agglutination.....	+++ to ++	+++ to ++	+++ to ++	++	+	sl. tr.	sl. tr.	—	—	sl. tr.
Resuspension.....	+++++	+++++	+++++	+++	+	0	0	—	—	0
Interface.....	+++++	+++++	+++++	+++	+	tr.	0	—	—	0
Cataphoresis, μ/sec	0.35	0.47	0.47	0.92	1.40	1.33	1.53	—	—	1.52
Phagocytosis, per cent.....	44	46	46	28	43	35	9	—	—	3

Series D. Bovine III in Anti-Bovine III Serum.

Agglutination.....	+++++ to ++++	+++++ to ++++	+++++ to ++++	+++++	++	sl. tr.	sl. tr.	sl. tr.	sl. tr.	sl. tr.
Resuspension.....	+++++	+++++	+++++	+++++	++	0	0	0	0	0
Interface.....	+++++	+++++	+++++	+++ to ++	++ to +	tr.	1.53	—	—	0
Cataphoresis, μ/sec	0.08	0.17	0.17	0.31	1.18	1.17	1.46	1.50	1.69	1.69
Phagocytosis, per cent.....	47	44	44	45	20	31	15	4	6	6



TEXT-FIG. 2. Homologous and cross reactions between an avian tubercle bacillus and a saprophyte and their corresponding rabbit immune sera. Sera aged about 16 months. Symbols as in Fig. 1. Note phagocytic prezones.



TEXT-FIG. 3. Homologous and cross reactions between frog and milk bacilli and their corresponding rabbit immune sera. Sera aged about 16 months, and filtered before use. Symbols as in Fig. 1. Note phagocytic prezones.

changes and enhanced phagocytosis, but again with a striking phagocytic prezone. This serum, on the other hand, failed to increase phagocytosis of Prague bacillus, although it caused moderate surface changes (Series B).

Anti-milk bacillus serum from the same source as in Text-fig. 2 was passed through a Seitz asbestos laboratory filter disc and used in the experiment represented by Text-fig. 3. It is seen that the percentage phagocytosis caused by the filtered serum is somewhat less than that caused by the unfiltered, and the phagocytic prezone has been almost abolished by the filtration (Series A). The milk bacillus serum was practically without effect either on the surface properties or the phagocytosis of frog bacillus (*M. ranæ*, Series C). Anti-frog-bacillus serum, which also had been filtered through a Seitz disc, caused surface changes and increased phagocytosis, with prezone, with its homologous antigen (Series D). The anti-frog bacillus serum caused moderate surface reactions but no increased phagocytosis with the heterologous organism (Series B).

Two conditions have been found, then, in which correspondence may fail between the effects produced by aged sera on the bacterial surface properties and on phagocytosis. Save for them the correspondence of surface and phagocytic effects has been satisfactory even with aged immune sera. These conditions are:

1. The aged sera in all of 13 homologous series gave phagocytic prezones. Maximum phagocytosis was reached in serum dilutions which might be as high as 1:1000; phagocytosis in the lesser serum dilutions was far below the maximum or even absent. Prezones comparable to this were not found with the surface reactions.

2. The aged sera sometimes produced surface changes but no phagocytosis when reacting with heterologous bacterial strains. Four out of 11 series gave this result; in the other 7 heterologous series correspondence between bacterial surface changes and phagocytosis was satisfactory.

The Phagocytic Prezone with Aged Immune Sera.—Phagocytic prezones occurred, as has been said, in all of the homologous series with the aged immune sera; only one prezone occurred in a heterologous series. Moreover we have two clear experiments in which an aged serum gave a striking prezone with its homologous bacterial strain,

but caused phagocytosis without prezone with a heterologous strain. The first example is given by Series A and C of Text-fig. 2. The second is given by Series A and C of Table II. Series B and D of Table II seem to be a third example, but this is less clear because of the irregularity of the phagocytosis in Series D.

The results just cited indicate that the phagocytic prezone cannot be chiefly due to injury of the leucocytes by the aged sera or to other non-specific effect, but must be an expression of the properties of the specific combination of bacteria and sensitizing substances. The evidence leading to this conclusion is strengthened by the observation that a phagocytic prezone may persist after washing the sensitized bacteria. In 4 series in which the sensitized bacteria were washed, some prezone persisted in 3; in the fourth series phagocytosis was practically abolished by washing. Two of the persistent prezones were, however, considerably diminished by the washing; one of these is shown in Table III.

It is important to bear in mind that these phagocytic prezones with aged sera, however interesting and perplexing from a theoretical standpoint, are a distinctly artificial phenomenon. Such prezones have practically not occurred, at least in our experience, with sera used within a few days of drawing the blood, whether the sera have been heated to 56°C. for 30 minutes or have been used unheated. Such fresh sera have almost invariably shown phagocytosis at or near maximum in the highest serum concentration used in this work, namely a final concentration of 25 per cent serum.

The most marked prezone found with freshly prepared sera occurred in Rabbit 20-02, at the end of its 3rd week of immunization with Arloing bacillus (1); the percentages of phagocytosis in the successive tubes in this experiment (the dilution factor was 0.25 as usual) were 67, 72, 89, 97, 81, 26, 10, 11, and control 11 per cent. This prezone was not present with the serum of the same rabbit the week before or after this experiment. Moreover the leucocytes were noted as agglutinated in the first tubes of this series; this probably explains the apparent prezone.

There are good grounds for supposing that the protective power of some immune sera depends largely upon their efficiency in promoting phagocytosis (3). The abnormal bacteriotropic behavior of the aged sera used in our experiments suggests a danger in dispensing old anti-bacterial sera for clinical use.

TABLE II.
*Homologous and Cross Reactions between M. avium (Arloing Strain) and M. tuberculosis (Bovine III Strain) and Their
 Corresponding Rabbit Immune Sera.*

Prezones in phagocytosis are shown in homologous reactions but not in cross reactions.

	Serum dilutions						Sodium chloride control
	1:4	1:16	1:64	1:256	1:1024	1:4096	1:16,384
Series A. Arloing in Anti-Arloing Serum.							
Agglutination.....	+++	+	+++	+++	+	0	0
Resuspension.....	+++ to ++	+++	+++	+++ to ++	+	tr.	0
Interface.....	++	+++	+++	++	+	tr.	0
Cataphoresis, $\mu/sec.$	1.27	1.73	1.80	1.96	1.65	1.91	1.64
Phagocytosis, <i>per cent.</i> ..	22	22	72	96	81	40	8
Series B. Arloing in Anti-Bovine III Serum.							
Agglutination.....	+++	+++	+++	++	0	0	0
Resuspension.....	+++ to ++	+++ to ++	+++	tr.	+	0	0
Interface.....	+++	++	+++ to +	+	+	0	0
Cataphoresis, $\mu/sec.$	0.95	1.20	1.47	1.81	1.80	0	0
Phagocytosis, <i>per cent.</i> ..	53	26	25	9	11	10	1.77
							—

Series C. Bovine III in Anti-Arloing Serum.

Agglutination.....	+	+	tr.	0	0	0	0
Resuspension.....	++	+	++	+	0	0	0
Interface.....	+++	++ to +++	++	++	tr.	0	0
Cataphoresis, μ/sec	1.54	1.16	1.15	1.52	1.26		1.54
Phagocytosis, per cent..	24	19	10	12	13	9	1

Series D. Bovine III in Anti-Bovine III Serum.

Agglutination.....	+	+	+	+	tr.	0	0
Resuspension.....	+	++	++	++	+	tr.	0
Interface.....	++	+++	+++	+++	+	tr.	0
Cataphoresis, μ/sec	0.97	0.94	1.11	1.09	1.14	1.25	1.64
Phagocytosis, per cent..	15	11	14	11.5	29	3	—

TABLE III.

The Effect on Surface Properties and on Phagocytosis of Washing Bacteria Sensitized with Aged Serum. M. avium (Arloing Strain) Was Sensitized with Homologous Aged Rabbit Serum. In Series A Determinations Were Made in the Presence of Serum Dilutions; in Series B Sensitized Bacteria Were Washed to Remove Free Serum.

It is seen that the intensity of the phagocytic prezone is diminished by washing the bacteria.

	Serum dilutions					Sodium chloride control	
	1:4	1:16	1:64	1:256	1:1024	1:4096	1:16,384
Series A (in Serum Dilutions)							
Agglutination	+++ to ++	+++ to ++	+++	+++ to +++	tr. to 0	0	0
Resuspension	++	++	+++	+++	+	tr.	0
Interface	+++	+++	+++	++	+	+	0
Cataphoresis, $\mu/sec.$	0.93	1.47	1.69	1.76	1.50	1.42	1.61
Phagocytosis, per cent.	25	28	69	90	78	45	8
Series B (Sensitized, Washed)							
Agglutination	—	—	—	—	—	—	—
Resuspension	+++ to ++	+++	+++	+++	+++ to ++	0	0
Interface	+++ to ++	+++	+++	+++	+++ to tr.	0	±
Cataphoresis, $\mu/sec.$	0.28	0.33	0.55	1.25	1.42	1.57	1.64
Phagocytosis, per cent.	55	54	62	65	85	73	—
Series C (Sensitized in 1:256 Serum, Then Made Up in Serum Dilutions)							
Phagocytosis, per cent.	30	29	68	87	93	93	85
							—

Opsonins.

Rabbit Sera.—With fresh, unheated sera of normal rabbits there has been excellent parallelism between the bacterial surface changes and opsonization. Bacterial cohesion has been increased, surface potential difference reduced, wetting properties altered and the bacteria have been prepared for phagocytosis. The serum concentrations required to give these effects are of course much higher than with immune sera.

The lowest concentrations of normal serum which promoted phagocytosis were for sensitized, washed bacteria: 1:5, 1:5, 1:5, 4:5, 1:5, 4:5, and 1:5; one series in which the bacteria were washed failed to give phagocytosis even after sensitization with 80 per cent serum. For bacteria exposed to leucocytes in the presence of fresh normal sera the titers were somewhat higher, i.e., 1:16, 1:16, 1:64 and 1:16. The resuspension and cataphoresis titers have run on the average a little higher than the phagocytosis titers, the interface titers about the same, although all titers with these concentrated normal sera have tended to be irregular.

With the sera of normal rabbits inactivated by heating to 56°C. for 30 minutes correspondence between bacterial surface changes and phagocytosis has not been good. In 7 series of bacteria sensitized with heated normal sera and washed, phagocytosis was not induced in any, whereas the surface changes were present, although to a less degree than after sensitization with unheated serum. In 28 series in which the bacteria were exposed to leucocytes in the presence of heated normal rabbit serum phagocytosis was induced in only about 16 series, whereas the bacterial surface properties were altered to some degree in practically all.

Human Sera.

Bacteria have been sensitized with fresh human sera, washed with 0.85 per cent sodium chloride solution, and tested for surface changes and for phagocytosis by rabbit leucocytes. The sera tested were from 7 non-tuberculous individuals, from 5 cases of pulmonary and 6 of bone or joint tuberculosis, as indicated in the protocols below. The characteristic changes in bacterial surface properties were regularly produced by these sera. Qualitatively the changes were not distinguishable by the surface reactions from those produced by rabbit sera. Quantitatively the bacterial surface changes varied with the different

sera, but were usually a little greater than those produced by normal unheated rabbit sera. Yet in no case was a clearly demonstrable increase in phagocytosis by rabbit leucocytes caused by sensitization with human serum.

Patients with Pulmonary Tuberculosis.—(Sera made available through kindness of Dr. I. Kaufman.)

A. L., female. Pulmonary tuberculosis, moderately advanced, moderate symptoms, sputum positive; tuberculosis complement fixation, ++++; Wassermann negative. Unheated and heated serum tested in dilutions of 1:4, 1:16 and 1:64 with *M. tuberculosis* (Bovine III).

E. G., female. Pulmonary tuberculosis, moderately advanced, slight symptoms, sputum positive; tuberculosis complement fixation, negative; Wassermann negative; tuberculin, 0.00001 cc. O. T., ++. Unheated serum tested in dilutions of 1:4, 1:16, 1:64 and 1:256 with Bovine III.

M. C., female. Pulmonary tuberculosis with apical râles; far advanced; severe symptoms; sputum positive. Unheated and heated serum tested in dilutions of 1:4, 1:16 and 1:64 with Bovine III.

B. R., female. Pulmonary tuberculosis with apical râles; far advanced, moderate symptoms. Tuberculosis complement fixation, negative; Wassermann negative. Unheated and heated serum tested in dilutions of 1:4, 1:16 and 1:64 with Bovine III.

G. H., female. Apical tuberculosis; tuberculosis complement fixation ++++; tuberculin, 0.00001 cc. O.T., +. Wassermann ++++. Unheated serum tested in dilutions of 1:4, 1:16 and 1:64 with Bovine III.

Surgical Tuberculosis.—(Sera made available through kindness of Dr. George Wagoner.)

Sera of A. A. and C. P. tested unheated in dilutions of 1:4, 1:16, 1:64, 1:256 and 1:1024. Other sera tested, unheated, in dilutions of 1:4 and 1:16. All sera tested, heated, in dilutions of 1:4. Antigen for all series, *M. tuberculosis* (Bovine III).

A. A., male. Pott's disease of 4th, 5th, 6th and 7th dorsal vertebræ.

C. P., male. Pott's disease of 5th and 6th dorsal vertebræ.

I. L., male. Lumbo-sacral spondylitis, probably tuberculous, numerous sinuses, pulmonary tuberculosis (?).

J. S. Tuberculous left hip with sinuses; old pulmonary tuberculosis.

A. S. Tuberculous spine and shoulder with sinuses.

H. S. Tuberculous right wrist (?).

Non-Tuberculous Individuals.—

Sera from 6 persons who came into the chest clinic but were shown by physical and laboratory examination to be free from clinical tuberculosis. Serum from a healthy university student. Tests as above with Bovine III or an avian tubercle bacillus as antigen.

These experiments failed to give evidence that any of the reactions used afforded a satisfactory means of distinguishing the sera of tuberculous from those of non-tuberculous individuals.

It is to be emphasized that the bacteria sensitized with human sera were in all cases washed before testing for phagocytosis in order to exclude the possibility of a direct toxic effect of human serum on the rabbit leucocytes. The leucocytes were shown to be capable of phagocytosis by other series in the same experiments in which sensitization was produced by rabbit sera. A further experiment was performed in which bacteria were first sensitized with unheated normal rabbit serum, then washed and resuspended in 1:500 human serum before testing for phagocytosis. This concentration of human serum was considerably higher than the amount which could have remained after washing of the bacteria sensitized even with the highest concentrations of human serum. Phagocytosis of the bacteria sensitized with rabbit serum occurred nevertheless in the presence of the 1:500 human serum.

The rabbit polymorphonuclear leucocytes can then differentiate with precision bacteria sensitized with human serum from those sensitized with rabbit serum, whereas our physical-chemical tests fail to make this differentiation. We believe phagocytosis to be dependent upon the spreading of the leucocyte about the bacterium primarily under the action of surface forces (1). This capacity of a leucocyte to differentiate the sensitizing substances of two mammalian species³ implies a very striking selectivity in the spreading or wetting requirements of the leucocyte, which, however, is not wholly without analogy. Thus sponges have been separated into their component cells by straining through cloth and the separated cells of different species have been mixed together. The cells creep about by amœboid motion until they meet and fuse with other cells of the same species. Syncytia composed of cells of each species but not of mixed species are

³ However, we have obtained abundant phagocytosis by rabbit leucocytes of tubercle bacilli sensitized with hyperimmune antituberculous goat sera and washed with saline. Robertson and his collaborators obtained phagocytosis of pneumococci by rabbit leucocytes suspended in rabbit serum to which human serum containing antipneumococcus antibodies had been added (3). Normal serum added to such rabbit serum leucocyte mixtures did not cause phagocytosis (3, 4).

thus formed (5). The spreading of fluids, in general, is known to be to some extent selective, and to depend both on the properties of the spreading surface and the surface spread upon (6).

In the experiments with normal rabbit sera, then, excellent correspondence has been obtained between changes in bacterial surface properties and opsonic effects so long as fresh, unheated sera have been used. With heated normal rabbit serum or with human serum, heated or unheated, surface changes have been produced but phagocytosis has often failed. It would seem then that the requirements for spreading of the leucocyte surface are so delicate that discrimination occurs between sensitizing substances deposited on the one hand by fresh immune rabbit sera and fresh unheated normal rabbit sera and on the other hand by aged immune sera, heated normal rabbit sera and human sera. The fact may once again be emphasized that the failures of correspondence between bacterial surface changes and phagocytosis have all been failures of phagocytosis, and have all been found under extremely artificial conditions. Correspondence between changes in bacterial surface properties and increase in phagocytosis has been excellent when conditions *in vitro* have been made to imitate as nearly as possible conditions *in vivo*.

CONCLUSIONS.

The work reported in this and in previous papers (1, 7) demonstrates the following relations for acid-fast bacteria and rabbit polymorphonuclear leucocytes:

1. The combination of a substance or substances present in fresh immune rabbit serum, heated or unheated, or in fresh unheated normal rabbit serum, with a substance or substances in the bacterial surface causes an increase in cohesiveness, decrease in surface potential difference and characteristic alteration in wetting properties of the bacteria, and *prepares the bacteria for phagocytosis*.

2. (a) The effective substance or substances in the serum may become so altered as the result of heating or aging that combination with the bacterial surface, while causing changes in bacterial surface properties indistinguishable by the present physical-chemical tests from these just mentioned, *may not lead to phagocytosis*, or may lead to phagocytosis with a prezone not paralleled by a prezone in the changes in surface properties.

(b) Sensitization of bacteria with human sera causes changes in surface properties similar to those caused by rabbit sera, but does not lead to phagocytosis by rabbit leucocytes. The spreading requirements of rabbit polymorphonuclear leucocytes are evidently highly selective.

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ON THE MECHANISM OF OPSONIN AND BACTERIOTROPIN ACTION.

III. THE DEVELOPMENT AND EFFECT OF THE ANTIBODIES FOUND IN EXPERIMENTAL TUBERCULOSIS OF RABBITS.

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This study is concerned with three questions: first, the development of circulating antibodies in primary tuberculosis in rabbits; second, the development of antibodies in rabbits reinfected with tubercle bacillus while still incompletely recovered from a primary tuberculosis; and third, as in the two foregoing studies, with the correlation between the effects on bacterial surface properties and on phagocytosis produced by the sera of the animals studied.

The animals followed were taken from among those used by M. B. Lurie in continuation of his study of the fate of tubercle bacilli in the organs of the rabbit (1). Bacteriological data on these rabbits will be published later by Lurie. The titers of the sera of three groups of rabbits have been followed, each containing primarily infected as well as reinfected individuals. The sera were tested in all cases with suspensions in 0.85 per cent sodium chloride solution of a living bovine tubercle bacillus, Bovine III. This is an avirulent and now slightly atypical strain¹ (2). The test suspensions were adjusted to match a turbidity standard. The methods used are those described in a foregoing paper (3), except that the interface reaction was omitted. The sera were heated for 30 minutes at 56°C. in all cases.

¹ It was not practicable to use the infecting strain (Bovine C) as routine antigen in these tests because of the danger of infecting the workers. In a special experiment, however, living Bovine C bacilli were shown to exhibit typical surface changes and increase in phagocytosis as a result of serum sensitization. For all practical purposes Bovine III was an homologous antigen.

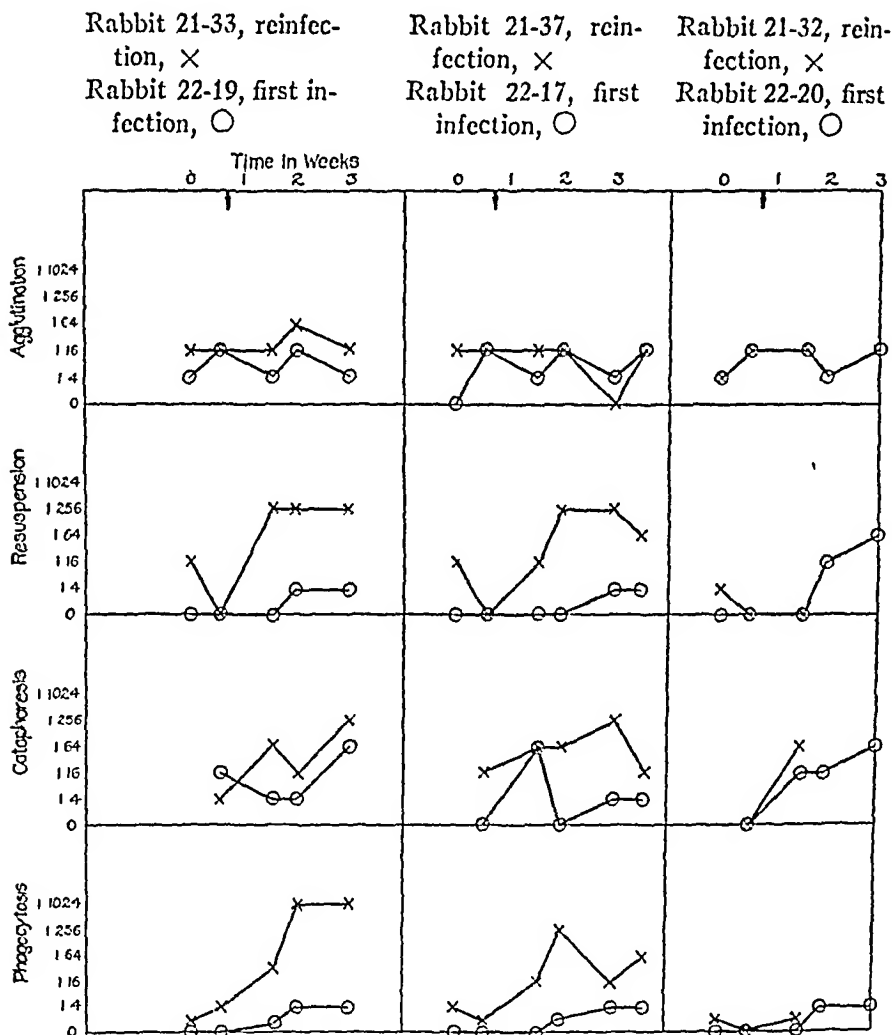


FIG. 1. Response in antibody production following a single injection (indicated by arrow at top of chart) of living, virulent tubercle bacilli. Three rabbits with residual foci of tuberculosis (crosses), and 3 previously healthy rabbits (circles). Ordinates represent the greatest dilutions of serum giving clearly positive reactions. It is shown that the reinfected animals promptly developed circulating antibodies strikingly in excess of those found in the course of primary infection. The changes in titer during tuberculous infection as indicated by the bacterial surface reactions (resuspension and cataphoresis) and by phagocytosis were in approximate correspondence.

Group 1.—Rabbits 21-32, 21-33 and 21-37 injected intravenously on May 17, 1928, with 0.001 mg. per kilo body weight of a living virulent human tubercle bacillus, Strain P-48A. X-ray of rabbits' lungs, Oct. 24, 1928, showed for Rabbit 21-32 slight, and for Rabbit 21-37 moderate disseminated pulmonary tuberculosis; lesions in Rabbit 21-33 were not certainly detectable by x-ray. These previously infected rabbits and normal Rabbits 22-17, 22-19, and 22-20 were injected intravenously on Nov. 6 with 0.01 mg. of a living virulent bovine tubercle bacillus, Bovine C. The titers of the sera of the rabbits of this group are given in Text-fig. 1; the injections on Nov. 6 are indicated by arrows at the top of the chart. The titers represent the greatest dilutions of serum giving clearly positive reactions; doubtful reactions and traces are omitted as in the first paper (3). Cataphoresis after sensitization with these relatively concentrated sera does not give a sharp end point; therefore in each experiment any value representing a change (decrease) of 20 per cent or more from the average cataphoretic velocity of the unsensitized bacteria of that experiment has arbitrarily been taken as positive.

The most striking feature of Text-fig. 1, and also of the charts following, is the difference in response to injection of the reinfection and first infection animals. The titers of the two groups do not differ greatly before the injections of Nov. 6, although those of the infected animals tend to be a little higher. But a marked difference appears at the first bleeding 6 days after injection; the titers of reinfection Rabbits 21-33 and 21-37 have clearly increased, whereas those of the first infection rabbits are essentially unchanged. Reinfection Rabbit 21-32, whose titers showed little change at the first bleeding on Nov. 12, was in bad condition at the time and died Nov. 13.

An increase in titers appeared in each of the first infection animals at the second (Nov. 15) or third (Nov. 22) bleeding, respectively 9 and 16 days after the injections. Although slight, this increase was so uniform in the 3 animals that we believe it to have been significant.

The potency of the sera in changing the properties of the bacterial surfaces (resuspension and cataphoresis reactions) and in promoting phagocytosis are parallel, within the experimental error, as was found to be the case with sera of animals under active immunization (3). The "agglutination" reaction, shown in the uppermost lines, does not render a satisfactory account of the changes in the sera shown by the other reactions. It has already been pointed out that the ordinary agglutination technique is not satisfactory with mammalian tubercle bacilli as antigen (4, 5), but that the resuspension reaction is a satisfactory detector of the binding of agglutinins by these bacilli. Our

experiments thus afford strong corroborative evidence of the parallelism of agglutinin and bacteriotropin content in sera (6, 7), not evidence contradicting such parallelism as might appear on first inspection of the charts.

The sera of Rabbits 21-33 and 21-37, 16 days after reinfection, *i.e.*, when active against Bovine III, were tested for specificity against *M. chelonci* (turtle bacillus). They did not react to a significant degree with *M. chelonci*.

The autopsy protocols of the rabbits of this group are given below.

Autopsies by M. B. Lurie.

Rabbit 21-32, died Nov. 13, 1928, *i.e.*, 7 days after *reinfection*. Lungs: moderate number of discrete, rather firm subpleural tubercles. Liver, spleen and bone-marrow: no tubercles. Kidney: discrete tubercles in cortico-medullary region.

Rabbit 21-37, killed Dec. 18, 1928, *i.e.*, 42 days after *reinfection*. Lungs: posterior portions have large caseous pus-containing cavities; anterior portions show discrete subpleural tubercles. Liver and bone-marrow: no tubercles. Spleen: enlarged, surface irregular, no clear-cut tubercles. Kidney: moderate number of cortical tubercles.

Rabbit 21-33, killed Jan. 8, 1929, *i.e.*, 63 days after *reinfection*. Lungs: a few scattered fibrocaseous tubercles, 2 to 3 mm. in diameter. Spleen: enlarged and mottled, no tubercles. Kidney, liver and bone-marrow: no tubercles.

Rabbit 22-20, killed Dec. 3, 1928, *i.e.*, 27 days after *first infection*. Lungs: numerous miliary tubercles about 2 mm. in diameter with punctate caseous foci. Liver: minute punctate tuberculous foci. Spleen: numerous subcapsular tubercles about 1 mm. in diameter. Kidney: moderate number of cortical tubercles about 1 mm. in diameter. Bone marrow: a few discrete tubercles.

Rabbit 22-17, killed Jan. 10, 1929, *i.e.*, 65 days after *first infection*. Lungs: anterior portions almost entirely consolidated (diffuse caseous pneumonia); posterior portions show massive conglomerate miliary tuberculosis; extensive communicating caseous foci. Liver: appears normal. Spleen: several tubercles 3 to 4 mm. in diameter. Kidney: moderate number of discrete cortical tubercles. Bone-marrow: no tuberculosis.

Rabbit 22-19, killed Jan. 11, 1929, *i.e.*, 66 days after *first infection*. Lungs: massive conglomerate miliary tubercles, 1 to 10 mm. in diameter. Spleen: about 4 times normal in size; numerous reddish subcapsular tubercles, 2 to 3 mm. in diameter. Liver: a few minute tubercles. Kidney: moderate number of cortical and corticomedullary tubercles. Bone-marrow: ill-defined tubercles.

Two of the reinfection rabbits, Nos. 21-32 and 21-37, showed a moderate number of discrete lesions in lungs and kidney, in all probability residual from the primary infection with tubercle bacilli of

human type. In the third reinfection rabbit, No. 21-33, the primary infection had been almost overcome, leaving only a few scattered fibro-caseous tubercles in the lung. All 3 animals were highly resistant

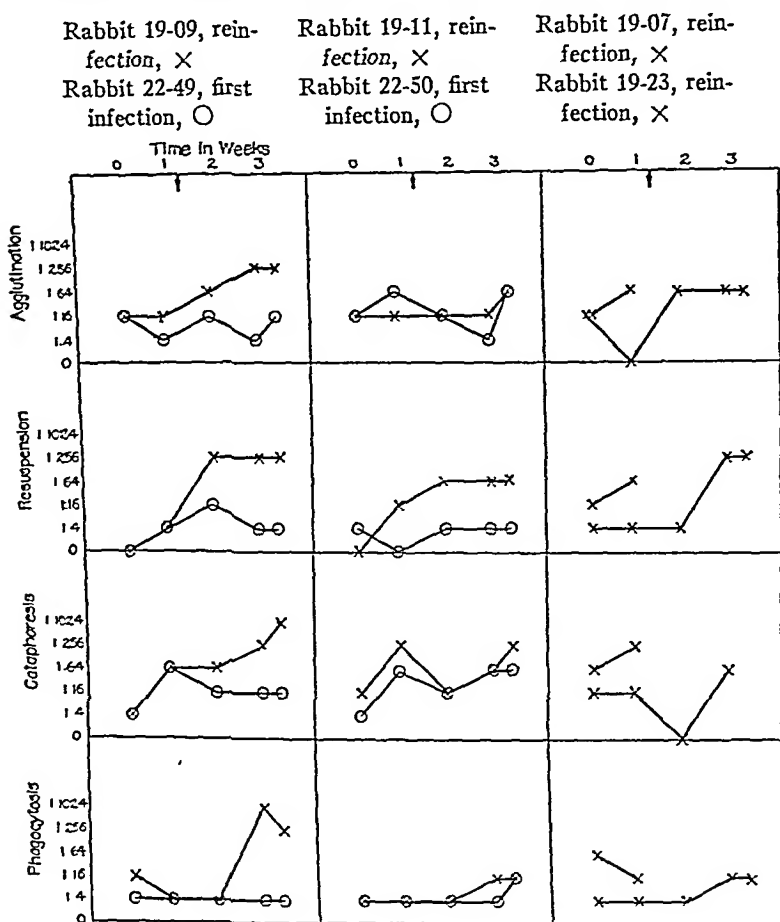


FIG. 2. Antibody production following single injection (indicated by arrow at top of chart) of living, virulent tubercle bacilli. Four rabbits with residual foci of tuberculosis (crosses), and 2 previously healthy rabbits (circles).

to the secondary infection with bovine tubercle bacilli; no lesions certainly referable to the second infection were found.

A marked contrast to the reinfection animals was afforded by the

rabbits infected with bovine tubercle bacilli without previous infection with bacilli of human type. In these the bovine infection ran its usual malignant course, causing after 2 months massive confluent tuberculosis of the lungs and involvement of the abdominal organs.

Group 2.—Rabbits 19-07, 19-09, 19-11 and 19-23 injected intravenously on Feb. 3, 1928, with 0.001 mg. per kilo body weight of a living, virulent, human tubercle bacillus, Strain P-48 A. These infected rabbits and normal Rabbits 22-49 and 22-50 injected intravenously on Oct. 11, 1928, with 0.01 mg. of living virulent bovine tubercle bacillus, Bovine C. Injections on Oct. 11 indicated in Text-fig. 2 by arrows at top of chart. In Groups 2 and 3 cataphoresis values are taken as positive if they represent a decrease of 10 per cent or more from the average cataphoretic velocity of the unsensitized bacteria of the experiment in question.

The increase in rate and quantity of antibody production in the reinfection rabbits as compared with the first infection rabbits again appears in Text-fig. 2. This difference is marked between Rabbits 19-09 and 22-49, less apparent between Rabbits 19-11 and 22-50. The reinfection rabbits in each case showed increase in titer by 11 days after injection; this increase was not consistently apparent 4 days after injection. No unequivocal increase in the titers of the 2 first-infection rabbits was detected within the 2 weeks after injection during which the experiment was continued.

Rabbit 19-07 died 3 days after reinfection. The titers before reinfection were unusually high. Also the amount of pulmonary disease was unusually great for rabbits infected with so small a dose of human bacilli. This association may have been coincidental, however.

The serum of Rabbit 19-11, 18 days after reinfection was tested for specificity against *M. chelonci*. It did not react significantly with *M. chelonci*.

Autopsies by M. B. Lurie.

Rabbit 19-07, died Oct. 14, 1928, *i.e.*, 3 days after reinfection. Lungs: extensive tuberculosis with cavitation; confluent subpleural lesions toward anterior margin. Pneumonia superadded in left lung. Liver: no tubercles. Spleen: no tubercles. Kidneys: 2 isolated tubercles in each.

Rabbit 19-09, killed Oct. 26, 1928, *i.e.*, 15 days after reinfection. Lungs: fibrocaseous consolidation of anterior margins. Lung elsewhere has a few discrete lesions. Liver, no tubercles. Spleen: enlarged and studded with firm grayish nodules. Kidney, no tubercles. Bone marrow: epiphysis of right tibia, caseous focus.

Rabbit 19-11, killed Nov. 8, 1928, *i.e.*, 28 days after *reinfection*. Lungs: moderate number of small glossy tubercles, 1 mm. in diameter, in both lungs; no caseation. Liver: fibrosis, but no tubercles. Spleen: enlarged; no gross tubercles. Kidneys: small number of pin-point tubercles. Bone marrow: no tubercles.

Rabbit 19-23, killed Nov. 23, 1928, *i.e.*, 43 days after *reinfection*. Lungs: numerous discrete, subpleural tubercles, 3 to 4 mm. in diameter with ill defined caseous centers. Liver: no tubercles. Spleen: no tubercles. Kidneys: small number of ill-defined cortical tubercles. Bone marrow: no tubercles. Fat depots remarkably well filled.

Rabbit 22-50, killed Nov. 21, 1928, *i.e.*, 41 days after *first infection*. Lungs: massive conglomerate miliary tubercles, 3 to 4 mm. in diameter, with marked caseation in centers. Liver: punctate tubercles. Spleen: extensive miliary tuberculosis. Kidney: moderate number of cortical tubercles with central punctate caseation. Bone marrow: moderate number of tubercles.

Rabbit 22-49, killed Dec. 12, 1928, *i.e.*, 62 days after *first infection*. Lungs: massive, conglomerate, miliary tubercles with conspicuous caseous centers; almost complete solidification. Liver: 1 or 2 questionable pin-point foci. Spleen: riddled with tubercles about 2 mm. in diameter. Kidneys: extensive cortical tubercles, 3 to 4 mm. in diameter, raised as hemispheres above the surface; tubercles have conspicuous caseous centers. Bone marrow: several discrete tubercles about 3 mm. in diameter. Animal well preserved.

Group 3.—Rabbits 16-13, 16-14, 16-20 and 16-22 injected intravenously on Oct. 17, 1927, with 0.001 mg. per kilo body weight of a living virulent human tubercle bacillus, Strain P-48A. Rabbits 16-13 and 16-14 and normal Rabbits 20-93 and 20-94 were injected intravenously on Apr. 18, 1928, with 0.01 mg. of living P-48A. Rabbit 16-22 and normal Rabbits 20-95 and 20-97 were injected intravenously on Apr. 18, and Rabbit 16-20 on Apr. 20, 1928, with 0.01 mg. of a living virulent bovine tubercle bacillus, Bovine C. Injections on Apr. 18 and 20 indicated in Text-figs. 3 and 4 by arrows at top of chart.

The rabbits of Group 3, (Text-figs. 3 and 4) were followed for a longer time, 6 or 7 weeks, after injection. Unfortunately the titrations made before injections are not available for comparison and are not indicated in the figures; human tubercle bacilli (P-48A), heated to 56°C. for 75 minutes, were used as antigen in the first titrations, and proved not to be satisfactory detectors. In the titrations plotted living Bovine III was used as antigen as in the other groups.

The increased antibody content of the sera of the reinfection rabbits as compared with the first infection rabbits is strikingly manifest in the majority of the titrations 7 days after reinfection and persists throughout the experiment. Although the titrations were quite irregular in this group, the difference between reinfection and first infection animals was brought out both by the bacterial surface re-

actions and by phagocytosis. Surface changes and phagocytosis again seem to be essentially concordant within the rather large experimental error.

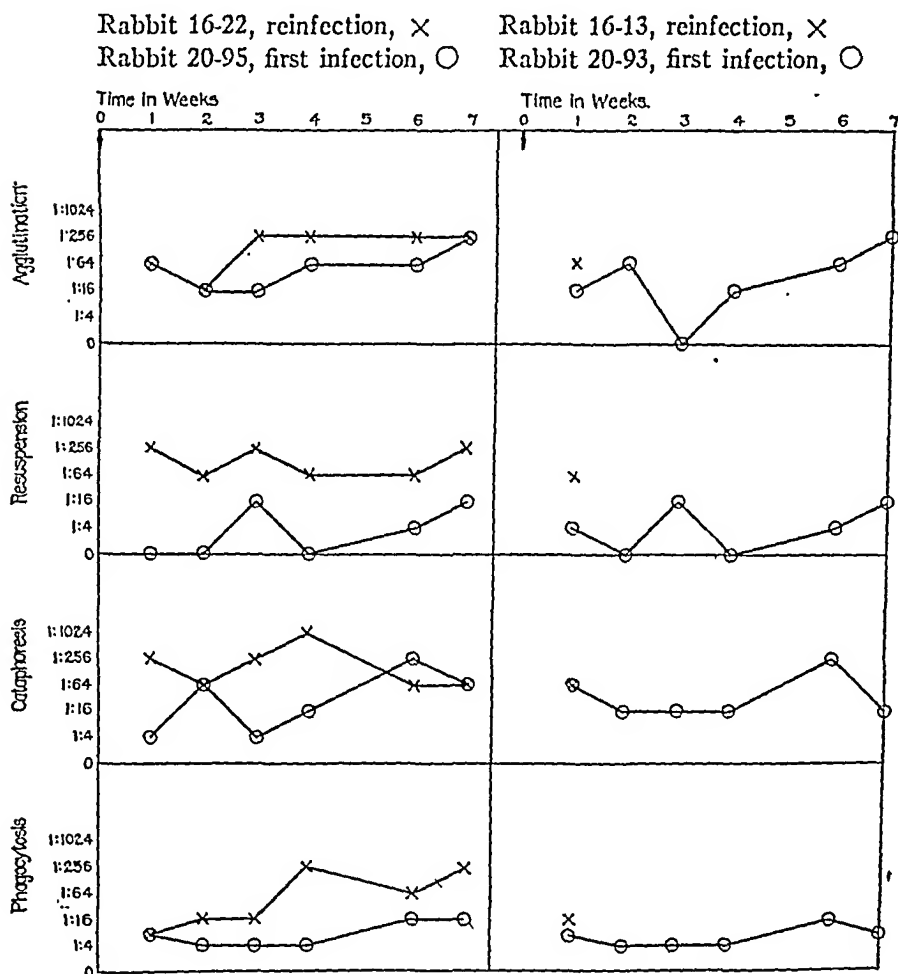


FIG. 3. Antibody production following a single injection (indicated by arrow at top of chart) of living, virulent tubercle bacilli. Two rabbits with residual foci of tuberculosis (crosses), and 2 previously healthy rabbits (circles).

The titers of the first infection animals seem to show a slight but nevertheless definite upward trend especially during the latter weeks of the experiment. Supporting data for this conclusion are afforded

by a comparison of the titrations of the sera of these first infection rabbits on the 6th and 7th week after infection with the titrations of 6 normal rabbits included in the same experiments. The results of

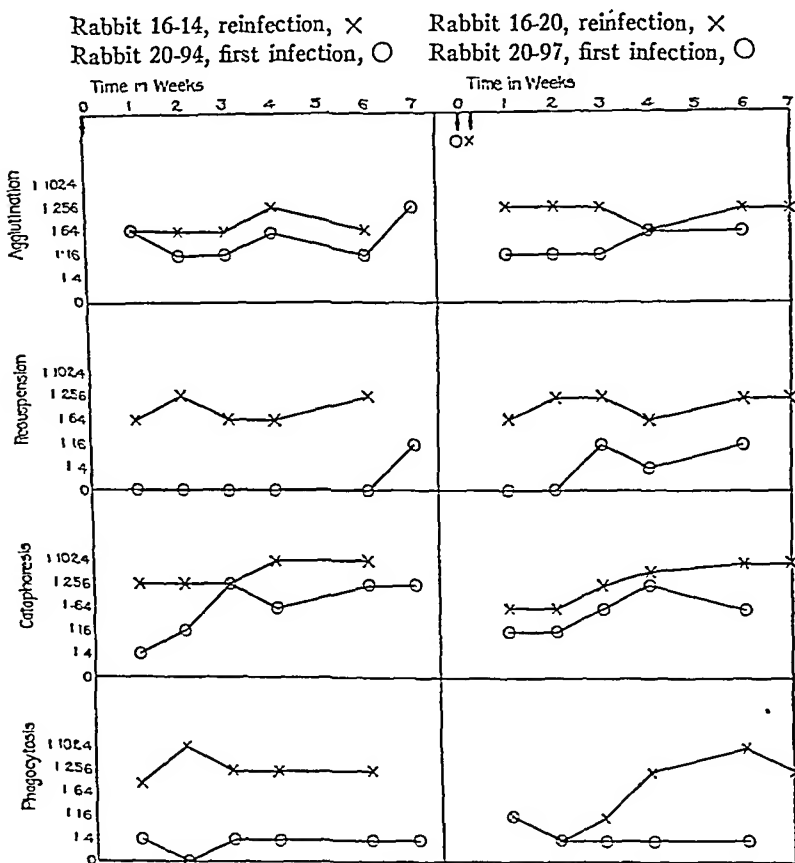


FIG. 4. Antibody production following a single injection of living, virulent tubercle bacilli. Two rabbits with residual foci of tuberculosis (crosses), and 2 previously healthy rabbits. Rabbit 16-20 injected 2 days later than the others (as indicated by arrow at top of chart).

these experiments are given in detail in Tables I and II. A definite phagocytosis-promoting power is present in the highest concentrations of serum from the first infection animals which is absent in the nor-

TABLE I.

Course of Tuberculous Infection (6th Week).

Details of experiment showing difference of response to virulent tubercle bacilli with respect to antibody formation. Three groups of rabbits are used: with residual foci of tuberculosis (reinfection), with primary infection (first infection), and normal animals, serving as controls.

	Serum dilutions					Sodium chloride control
	1:4	1:16	1:64	1:256	1:1024	

Reinfection Rabbit 16-14.

Agglutination.....	++ to +	++ to +	++ to +	tr.	0	0
Resuspension.....	+++	++	++ to +	+	0	0
Cataphoresis.....	0.56	0.95	1.18	1.37	1.47	1.69
Phagocytosis.....	39	18	16	10	7	4

Reinfection Rabbit 16-20.

Agglutination.....	++ to +	++ to +	++ to +	+	0	0
Resuspension.....	+++	++	+	+	0	—
Cataphoresis.....	0.82	0.97	1.22	1.08	1.46	1.65
Phagocytosis.....	27	16	19	13	10	4

Reinfection Rabbit 16-22.

Agglutination.....	++ to +	++ to +	++ to +	+	sl. tr.	1.54
Resuspension.....	++	++ to +	+	0	0	
Cataphoresis.....	0.78	0.93	1.23	1.68	1.67	
Phagocytosis.....	26	18	14	7	7	

First Infection Rabbit 20-93.

Agglutination.....	++ to +	+	+	sl. tr.	1.74
Resuspension.....	+	sl. tr.	0	0	
Cataphoresis.....	1.19	1.21	1.33	1.46	
Phagocytosis.....	14	10	4	3	

First Infection Rabbit 20-94.

Agglutination.....	++ to +	+	tr.	sl. tr.	1.55
Resuspension.....	tr.	0	0	0	
Cataphoresis.....	1.20	1.33	1.32	1.43	
Phagocytosis.....	12	5	3	2	

TABLE I—*Continued.*

	Serum dilutions				Sodium chloride control
	1:4	1:16	1:64	1:256	
First Infection Rabbit 20-95.					
Agglutination.....	++ to +	++ to +	+	sl. tr.	1.71
Resuspension.....	+	0	0	0	
Cataphoresis.....	1.20	1.38	1.36	1.47	
Phagocytosis.....	12	10	3	1	
First Infection Rabbit 20-97.					
Agglutination.....	++ to +	++ to +	+ to tr.	sl. tr.	
Resuspension.....	++ to +	+ to tr.	0	0	
Cataphoresis.....	1.31	1.34	1.40	1.63	
Phagocytosis.....	10	5	1	3	
Normal Rabbit 21-28.					
Agglutination.....	+	+	+	sl. tr.	
Resuspension.....	0	0	0	0	
Cataphoresis.....	1.11	1.42	1.42	1.32	
Phagocytosis.....	2	4	1	3	
Normal Rabbit 21-56.					
Agglutination.....	++ to +	++ to +	tr.	0	
Resuspension.....	+	tr.	0	0	
Cataphoresis.....	1.15	1.35	1.43	1.54	
Phagocytosis.....	4	3	3	1	

mal sera. A very slight and indefinite difference in the same direction is suggested in the titers of the other tests. The loss of phagocytosis-promoting power of heated normal rabbit serum, which, however, is still able to alter the bacterial surfaces, has been discussed in the second paper of this series (8).

The sera of Rabbits 16-20, 16-22, 20-93, 20-94 and 20-95 were tested on June 6, in serial dilution for specificity against typhoid bacilli. They did not agglutinate the typhoid bacilli, which were shown to be agglutinated by anti-typhoid serum.

The autopsies in this group are in essential accord with those in Groups 1 and 2 and need not be given in detail. The reinfection

TABLE II.

Course of Tuberculous Infection (7th Week). See Legend of Table I.

The experiment here shown gives details of results obtained 1 week after the experiment presented in Table I.

	Serum dilutions						Sodium chloride control
	1:4	1:16	1:64	1:256	1:1024	1:4096	
Reinfection Rabbit 16-20.							
Agglutination.....	++ to +	++ to +	++ to +	+	0	0	0
Resuspension	+++	+++	++	++ to +	0	0	0
Cataphoresis.....	0.88	1.07	1.38	1.28	1.34	1.61	1.62
Phagocytosis.....	37	17	14	13	6	2	1
Reinfection Rabbit 16-22.							
Agglutination.....	++ to +	++ to +	++ to +	+	0	0	0
Resuspension.....	+++	+++	++	++ to +	sl. tr.	sl. tr.	0
Cataphoresis.....	0.67	0.90	1.28	1.50	1.52	1.59	1.35
Phagocytosis.....	37	21	19	10	5	1	2
First Infection Rabbit 20-93.							
Agglutination.....	+	++ to +	++ to +	++ to tr.	0		
Resuspension.....	+++	++ to +	sl. tr.	sl. tr.	sl. tr.		
Cataphoresis.....	1.32	1.27	1.58	1.51	1.42		1.57
Phagocytosis.....	15	7	4	6	3		
First Infection Rabbit 20-94.							
Agglutination.....	++ to +	++ to +	++ to +	++ to tr.	tr.		
Resuspension.....	++	+	sl. tr.	tr.			
Cataphoresis.....	1.26	1.28	1.36	1.36	1.41		1.34
Phagocytosis.....	10	4	2	1	3		
First Infection Rabbit 20-95.							
Agglutination.....	++	++ to +	+	++ to tr.	tr.		
Resuspension.....	++ to +	+	sl. tr.	sl. tr.	sl. tr.		
Cataphoresis.....	1.09	1.44	1.28	1.53	1.31		1.80
Phagocytosis.....	13	10	5	2	1		

TABLE II—*Continued.*

	Serum dilutions			
	1:4	1:16	1:64	1:256
Normal Rabbit 21-69.				
Agglutination.....	+	+	+	0
Resuspension.....	++ to +	+	sl. tr.	sl. tr.
Cataphoresis.....	0.91	1.27	1.35	1.39
Phagocytosis.....	6	2	5	
Normal Rabbit 21-73.				
Agglutination.....	+	++ to +	+	+ to tr.
Resuspension.....	+ to tr.	+ to tr.	sl. tr.	sl. tr.
Cataphoresis.....	1.32	1.30	1.36	1.42
Phagocytosis.....	4	2	4	
Normal Rabbit 21-75.				
Agglutination.....	++	++ to +	tr.	sl. tr.
Resuspension.....	++ to +	+	tr.	sl. tr.
Cataphoresis.....	1.09	1.23	1.39	1.38
Phagocytosis.....	2	3	0	
Normal Rabbit 21-77.				
Agglutination.....	+	+	+	+ to tr.
Resuspension.....	+	+ to tr.	sl. tr.	sl. tr.
Cataphoresis.....	1.05	1.28	1.36	1.53
Phagocytosis.....	5	3		

animals showed some residual tuberculosis in the lungs and kidneys, but no evidence of lesions due to the second infection. In the primarily infected rabbits, the infection ran its typical course.

DISCUSSION.

An augmented capacity of animals to produce antibodies against antigens to which they had previously been immunized was demonstrated by Cole in 1904.

Cole (9) immunized rabbits by intravenous injection of living typhoid bacilli and kept them until the resulting serum agglutinin titers had fallen to low values. Such rabbits on reinoculation with typhoid bacilli promptly produced antibodies in

considerable quantity in response to dosages which produced only negligible effects in normal rabbits. V. Liebermann and Acél (10) later demonstrated an augmented response in agglutinins and bactericidal antibodies in guinea pigs reinjected with typhoid bacilli. Dean and Webb (11) have reported the production of high titer antihorse sera in rabbits by two or more courses of injections at 2 or 3 months intervals. A single course of 8 or 10 or more injections gave sera of inferior titers. Schneider (12) revaccinated (with vaccinia virus) rabbits whose antiviral titers from the first vaccination had fallen to low values. Although revaccination led only to negligible visible symptoms, prompt and considerable increase in antiviral titers was demonstrated. Opie and Freund (13) found that the production of precipitin against crystalline egg albumin was accelerated and augmented in rabbits which had previously been immunized with crystalline egg albumin and had no precipitins in their blood at the time of reimmunization. It has also been found that antibody titers in previously vaccinated or infected men or animals may rise in response to heterologous antigens (14, 15, 16, 17), a phenomenon termed by Conrad and Bieling the "anamnestische Reaktion."

The most detailed account of these phenomena now available seems to be that of Bieling (18), who studied the production of antibodies in rabbits in response to injections of typhoid and of dysentery bacilli. Antibodies corresponding to the antigen first injected reappeared on later injection of either homologous or heterologous antigen; heterologous antibodies reappeared at once, homologous antibodies after a latent period.

One feature of the reactions described by Bieling suggests a relationship of these phenomena to that described by Lewis and Loomis (see below). According to Bieling (18) rabbits previously immunized with dysentery bacilli formed typhoid agglutinins in response to small injections of typhoid bacilli which were without effect in normal animals. At the same time the dysentery agglutinins again increase. The first specific immunization leaves behind therefore an increased capacity to respond to later injections which is non-specific.

It has been established, then, that an animal which has once produced antibodies in response to a specific antigen may reproduce these antibodies more quickly or in greater amount or both in response to a later injection of the homologous antigen, and may even produce such antibodies in response to injection of a heterologous antigen.² It seems clear that this phenomenon is at least a factor in the augmented

²In the present study the preliminary and reinfecting antigens used were essentially homologous. For although tubercle bacilli of human type were necessarily used for the preliminary infection, whereas reinfection was in most instances with bovine bacilli, human and bovine tubercle bacilli have repeatedly been found to be serologically indistinguishable. See (2) in which earlier references are cited.

production of antibodies against the tubercle bacillus which we have demonstrated in animals with residual tuberculous foci.

A phenomenon which may in part rest upon a common basis with the "anamnestic reaction" and which doubtless contributed to the augmented antibody production in our reinfection rabbits has been described by Lewis and Loomis (19). These authors found in tuberculous animals a greatly increased capacity for antibody production in response to a cellular and also a bacillary antigen. Other conditions than tuberculosis also provoked to some degree this heightened "allergic irritability."

In the present experiments there has been found a slight and slowly developing production of antibodies in first infection rabbits which showed (20) a moderate and slowly developing resistance to tubercle bacilli, and a prompt production of antibodies in quantity in reinfection animals found (20) to be highly resistant to the reinfecting tubercle bacilli.

The relation of antibodies to resistance in tuberculosis has recently been discussed by Opie (21). The present experiments suggest that an increased capacity to produce antibodies may be a factor in the heightened resistance of previously infected tuberculous animals. However, definite conclusions cannot be drawn at the present time.

The chief purpose for which these experiments were conducted was the testing under still another set of conditions of the relation between the changes brought about by sera in the surface properties and the phagocytosis of bacteria. The experimental error in these titrations was somewhat larger in proportion to the titers themselves than in the foregoing papers, but was not so large as to invalidate the interpretation of the results, even in the case of first infection animals. Within this error, complete correlation was again found between the effects of the sera in modifying the bacterial surface properties and in promoting phagocytosis, except in the case of heated normal sera; this exception had already been discussed. In experimental tuberculosis, as in the other conditions studied, whenever a serum has promoted phagocytosis it has produced in roughly corresponding degree the characteristic alterations in the bacterial surfaces,—and, with the exceptions noted,—whenever the surface changes have been produced phagocytosis has been promoted.

We believe that we have shown, then, that serum sensitization of acid-fast bacteria consists in or involves the combination with and deposition on the bacterial surface of a component or components which increase the cohesion, reduce the surface charge, alter the wetting properties and promote the phagocytosis of the bacteria. Further analysis of the process is in progress.

Throughout this work we have had able technical assistance from Mr. H. J. Henderson.

SUMMARY.

Rabbits infected intravenously with virulent mammalian tubercle bacilli have in a majority of cases developed circulating antibodies to a slight but appreciable degree. The increase in titer was detected in one group of rabbits within the 2nd or 3rd week, in others during the 2nd month of infection.

Rabbits with residual pulmonary foci, resulting from infection 6 months or more previously with human tubercle bacilli, on reinfection with bovine tubercle bacilli promptly developed circulating antibodies strikingly in excess of those found during the course of primary infection. Such antibodies were present 6 or 7 days after reinfection.

The changes in titer during tuberculous infection as detected by the bacterial surface reactions and by phagocytosis were again, within the experimental error, in quantitative correspondence. The loss of phagocytosis-promoting power in heated normal serum involves an exception to this correspondence between surface and phagocytosis effects. This exception has already been discussed in an earlier paper (8).

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TRANSMISSION OF RESPIRATORY ANAPHYLAXIS (ASTHMA) FROM MOTHER TO OFFSPRING.*

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In the past it has been thought that nasal sensitization could be brought about only through the insufflation of solutions. Through work recently reported we have proved that nasal sensitization can be induced by the inhalation of a dry substance (horse dander). We have demonstrated¹ that when guinea pigs are subjected to an organic dust-laden atmosphere, they become sensitized, after a certain incubation period, to the substance which they have inhaled. We have termed this type of hypersensitiveness "respiratory anaphylaxis."

In a further series of studies² it was shown that a guinea pig sensitized by parenteral injection prior to or during pregnancy will sensitize her offspring in utero—passively or actively—and that this mechanism is traceable to the permeability of the placenta.

It has now become of interest to determine whether a pregnant animal made sensitive by inhalation, can transmit sensitizing substances to her fetus in utero.

EXPERIMENTS.

For these experiments, mature female guinea pigs were kept in our animal house for a number of weeks. In no instance was any evidence

* Preliminary report, *Proc. Soc. Exper. Biol. & Med.*, 1928, xxvi, 8. This work is being carried on under "The Crane Research Fund for the Study of Allergic Diseases in Children."

¹ Ratner, B., Jackson, H. C., and Gruehl, H. L., *Proc. Soc. Exper. Biol. & Med.*, 1925, xxiii, 16, 17; *Am. J. Dis. Child.*, 1927 xxiv, 23. Ratner, B., and Gruehl, H. L., *Proc. Soc. Exper. Biol. & Med.*, 1928, xxv, 661.

² Ratner, B., Jackson, H. C., and Gruehl, H. L., *Proc. Soc. Exper. Biol. & Med.*, 1926, xliii, 327; *J. Immunol.*, 1927, xiv, 249, 267, 275, 291, 303.

of dyspnea observed. After this period of observation, the animals were placed in cages with normal males for mating and then exposed to horse dander dust—in a manner previously described³—during various periods of time. In certain instances conception occurred prior to the period of sensitization. Hence it came about that we had mothers which had been sensitized to horse dander dust either before or during pregnancy. After varying intervals of time, these pregnant animals were again placed in contact with horse dander dust and in the majority of instances they manifested definite respiratory anaphylaxis (asthma).

Soon after birth, the offspring of these mothers were brought into contact with horse dander dust and their reactions on first contact with this substance noted. 1 hour later both the mothers and their offspring were given an intravenous injection of an alkaline extract of this horse dander,⁴ in order to test whether such respiratory disturbances as had been observed were anaphylactic in nature. The details of our experiments are given in the following protocols.

PROTOCOLS.

Family 1.

Female 1030 (510 gm.) was placed in the inhalation chamber and exposed to the horse dander dust for a total of 9 hours between Nov. 21, 1927 and Dec. 1, 1927; in no instance was there any evidence of dyspnea. On Feb. 16, 1928, or 2½ months later, she was placed in the inhalation chamber and showed severe convulsions and marked dyspnea—definite respiratory anaphylaxis. At varying intervals of time she was again brought into contact with this dust and exhibited definite symptoms of respiratory anaphylaxis. On May 10, 1928 she gave birth to 2 offspring.

Offspring 1454, 1455 (90, 100 gm.), when 5 days old, on May 15, 1928, each received an intravenous injection of 0.5 cc. dander extract and both died in typical anaphylactic shock.

Mother on the same day was given 0.5 cc. dander extract intravenously and also died in typical anaphylactic shock with lungs typically ballooned.

³ Ratner, B., Jackson, H. C., and Gruehl, H. L., *Am. J. Dis. Child.*, 1927, xxxiv, 23.

⁴ Ratner, B., Jackson, H. C., and Gruehl, H. L., *Am. J. Dis. Child.*, 1927, xxxiv, 23.

Family 2.

Female 1311 (640 gm.) was sensitized by exposure to horse dander dust for 8 hours from Jan. 20, 1928 to Jan. 28, 1928. On Feb. 13, 1928, 24 days after her initial exposure, she gave birth to 1 pig.

Offspring 1344 when 4 days old was placed in the inhalation chamber and showed definite evidences of moderate respiratory anaphylaxis.

Mother.—This guinea pig again became pregnant shortly after the previous confinement and at varying intervals, on being placed in the inhalation chamber, showed evidences of marked respiratory anaphylaxis. The second litter of 3 offspring was born on July 11, 1928.

Offspring—second litter—1516, 1517, 1518 (65, 70, 85 gm.) were placed in the inhalation chamber when 1 day old and all had marked respiratory anaphylaxis. This respiratory anaphylaxis was much more severe than that exhibited by the offspring of the first confinement. On the same day each received an intravenous injection of 0.3 cc. dander extract and all died in typical anaphylactic shock.

Mother (795 gm.) on the same day manifested marked respiratory anaphylaxis when placed in the inhalation chamber and on intravenous injection of 0.5 cc. dander extract showed profound anaphylaxis, dyspnea and collapse with final recovery.

Family 3.

Female 1509 (745 gm.) was exposed to horse dander dust for 6 hours in all from May 31, 1928 to June 6, 1928 and showed no signs of dyspnea during the exposures. 3 weeks after the initial exposure, on June 20, 1928, she was again placed in the inhalation chamber and showed marked respiratory anaphylaxis. This respiratory anaphylaxis was manifested at another contact with horse dander. On July 26, 1928, a little less than 2 months after the initial sensitizing exposure, the animal gave birth to 2 offspring.

Offspring 1594 and 1595 (80 gm. each) when 1 day old were placed in the inhalation chamber and manifested marked symptoms of respiratory anaphylaxis. On the same day each received 0.3 cc. dander extract intravenously following which both died in typical anaphylactic shock.

Mother on the same day, when placed in the inhalation chamber, exhibited moderate respiratory anaphylaxis and on intravenous injection of 0.5 cc. dander extract showed unmistakable signs of anaphylaxis—collapse, dyspnea, suffusion of the eyes, with final recovery.

Families 4 and 5 gave similar results.

Family 6.

Female 1217 (550 gm.) was sensitized in the inhalation chamber for 9 hours from Nov. 21, 1927 to Dec. 1, 1927 with no evidences of dyspnea. When again placed in the inhalation chamber on Feb. 16, 1928 she showed marked respiratory

anaphylaxis. This respiratory anaphylaxis was repeated at various intervals whenever the animal was placed in the inhalation chamber. On Sept. 3, 1928 she gave birth to 2 offspring.

Offspring 1626, 1627 (125, 120 gm.) when 10 days old, on Sept. 13, 1928, were placed in the inhalation chamber for the first time and showed marked evidences of respiratory anaphylaxis. Offspring 1626 gave a very profound reaction. It recovered, however, and after an intravenous injection of 0.3 cc. dander extract it showed only marked anaphylaxis with recovery. On the other hand Offspring 1627, which was not so profoundly affected in the inhalation chamber, died in acute anaphylactic shock after an intravenous injection of 0.3 cc. dander extract.

Mother on the same day showed moderate respiratory anaphylaxis in the inhalation chamber and after an intravenous injection of 0.5 cc. dander extract exhibited definite anaphylaxis with recovery.

Family 7.

Female 1466 (740 gm.) was sensitized in the inhalation chamber for 3½ hours from May 24, 1928 to May 29, 1928 and showed no dyspnea at all. When again placed in the inhalation chamber on June 13, 1928, 3 weeks after the initial exposure, she had no evident reaction. On June 28, 1928, she gave birth to 2 offspring.

Offspring 1512, 1513 (60, 65 gm.) when 12 hours old were placed in the inhalation chamber and showed profound respiratory anaphylaxis. Intravenous injection of 0.3 cc. dander extract produced death in Offspring 1512 and marked anaphylaxis—convulsions, collapse, dyspnea—with recovery in Offspring 1513.

Mother.—It is interesting that the mother when placed in the inhalation chamber on the same day was still negative and showed only slight symptoms of anaphylaxis after an intravenous injection of 0.5 cc. dander extract.

Family 8.

Female 1467 (610 gm.) was placed in the inhalation chamber for 3½ hours from May 24, 1928 to May 29, 1928 and showed no signs of respiratory anaphylaxis. On June 13, 1928, after an incubation period of 3 weeks, when again placed in the inhalation chamber, she was negative. 2 weeks after this, however, when placed in the inhalation chamber she demonstrated marked respiratory anaphylaxis. On July 21, 1928, a little less than 2 months after the beginning of sensitization, she gave birth to 3 offspring.

Offspring 1583, 1584, 1585 (50, 85, 75 gm.) when 3 days old, on July 24, 1928 showed moderate dyspnea when placed in the inhalation chamber and moderate signs of anaphylaxis after an intravenous injection of 0.3 cc. dander extract.

Mother on the same day in the inhalation chamber and after an intravenous injection of 0.7 cc. dander extract, showed the same moderate symptoms as did the offspring.

Families 9, 10, 11 and 12 gave similar results.

Family 13.

Female 1460 (700 gm.) was exposed to dander in the inhalation chamber for $\frac{1}{2}$ hour on May 23, 1928 and $\frac{1}{2}$ hour on May 24, 1928 and showed no dyspnea. This animal gave birth to 2 offspring on May 25, 1928, the 2nd day after the initial exposure.

Offspring 1482, 1483 (215, 210 gm.) when 27 days old, on June 21, 1928, were both put in the inhalation chamber. Offspring 1482 showed marked respiratory anaphylaxis and after intravenous injection of 0.5 cc. dander extract died in typical anaphylactic shock. Offspring 1483 was practically negative in the cage but after an intravenous injection of 0.5 cc. dander extract manifested moderate anaphylaxis with recovery.

Mother on the same day—28 days after her initial exposure to dander—was placed in the inhalation chamber and was negative. On the same day she showed definite anaphylaxis with recovery after an intravenous injection of 0.5 cc. dander extract.

Family 14.

Female 1265 (400 gm.) was exposed to dander in the inhalation chamber for 11 hours from Dec. 22, 1927 to Jan. 13, 1928 with no signs of dyspnea. On Jan. 16, 1928, 25 days after the initial exposure to dander, she gave birth to 2 offspring.

Offspring 1308, 1309 (190, 150 gm.) on Feb. 7, 1928, when 22 days old, were not placed in the inhalation chamber but were each given an intravenous injection of 0.5 cc. dander extract. Offspring 1308 was practically negative and Offspring 1309 showed only suffusion of the eyes.

Mother on the same day, however, when given an intravenous injection of 0.5 cc. dander extract had convulsions, collapse, marked dyspnea and died in typical anaphylactic shock after collapse for about $\frac{1}{2}$ hour.

Family 15.

Female 1173 (540 gm.) was exposed to dander in the inhalation chamber for 10 hours from Nov. 7, 1927 to Nov. 19, 1927 with no signs of dyspnea. On Feb. 16, 1928, 3 $\frac{1}{2}$ months after the initial exposure, when again placed in the inhalation chamber, this animal showed definite respiratory anaphylaxis. She was placed in the inhalation chamber at 2-week intervals throughout her pregnancy and each time exhibited marked respiratory anaphylaxis. On July 13, 1928 this animal gave birth to 1 offspring.

Offspring 1541 (130 gm.) on July 19, 1928, when 6 days old, showed moderate dyspnea in the inhalation chamber and after intravenous injection of 0.3 cc. dander extract had marked anaphylaxis with collapse and final recovery.

Mother on the same day was negative in the inhalation chamber and also after intravenous injection of 0.5 cc. dander extract.

Family 16.

Female 1461 (580 gm.) was exposed to dander in the inhalation chamber for 4 hours from May 23, 1928 to May 29, 1928, with no evidence of dyspnea. On June 8, 1928, 16 days after the initial contact with dander, she gave birth to 3 offspring.

Offspring 1493, 1494, 1495 (120, 130, 130 gm.) when 13 days old, on June 21, 1928, were placed in the inhalation chamber for the first time and were negative. Each animal received an intravenous injection of 0.5 cc. dander extract on the same day; Offspring 1493 was negative, Offspring 1494 and 1495 each showed moderate anaphylaxis with recovery.

Mother on the same day was placed in the inhalation chamber and had typical respiratory anaphylaxis. After an intravenous injection of 0.5 cc. dander extract she showed typical anaphylaxis with recovery.

Family 17.

Female 1408 (840 gm.) was exposed to dry horse dander for 6 hours from Mar. 15, 1928 to Mar. 21, 1928 and showed no signs of dyspnea. This sensitization was prior to pregnancy. This animal was placed in the inhalation chamber at 2-week intervals and was negative until about 1½ months before confinement when she began to develop symptoms of moderate respiratory anaphylaxis. On July 30, 1928, 4 offspring were born.

Offspring 1596, 1597, 1598, 1599 (80, 60, 75, 70 gm.) when 1 day old, on July 31, 1928, were placed in the inhalation chamber and all showed only suggestive symptoms of respiratory anaphylaxis. On intravenous injection of 0.3 cc. dander extract each again showed only suggestive symptoms.

Mother on the same day when placed in the inhalation chamber and also after intravenous injection of 0.5 cc. dander extract demonstrated only suggestive symptoms.

Family 18.

Female 1463 (600 gm.) was placed in the inhalation chamber for 3½ hours from May 24, 1928 to May 29, 1928 with no symptoms of dyspnea. On June 18, 1928, 25 days after the initial exposure, when again placed in the inhalation chamber she showed signs of moderate dyspnea but when subsequently placed in the inhalation chamber at various intervals, there were no signs of dyspnea. On July 24, 1928, 2 months after the beginning of sensitization, 3 offspring were born.

Offspring 1586, 1587, 1588 (75, 70, 75 gm.) were placed in the inhalation chamber when 2 days old, on July 26, 1928, and gave a slight to negative reaction. On the same day an intravenous injection of 0.3 cc. dander extract brought forth no anaphylactic reaction.

Mother on the same day showed no symptoms of anaphylaxis after exposure to dander and none after intravenous injection of 0.6 cc. dander extract.

Family 19.

Female 1469 (520 gm.) was placed in the inhalation chamber for 3½ hours from May 24, 1928 to May 29, 1928 and showed no dyspnea. On June 7, 1928 she gave birth to 2 offspring.

Offspring 1491, 1492 (55, 35 gm.) were placed in the inhalation chamber when 1 day old, on June 8, 1928, and were both negative. They showed no signs of anaphylaxis after an intravenous injection of 0.3 cc. dander extract.

Mother was negative when placed in the inhalation chamber on the same day and also after an intravenous injection of 1 cc. dander extract.

Families 20, 21 and 22 gave similar results.

Family 23.

Female 1473 (510 gm.) was placed in the inhalation chamber for 6 hours from May 31, 1928 to June 6, 1928 with no signs of dyspnea. On June 20, 1928, 20 days after the initial contact, she was again placed in the inhalation chamber and was negative. On June 23, 1928, 2 offspring were born.

Offspring 1494, 1495 (60, 55 gm.) were placed in the inhalation chamber on June 26, 1928, when 3 days old, and were negative. They were also negative after an intravenous injection of 0.5 cc. dander extract.

Mother on the same day was negative in the inhalation chamber and after intravenous injection of 0.5 cc. dander extract.

Families 24, 25 and 26 gave similar results.

ANALYSIS OF PROTOCOLS.

As controls for these experiments we had 11 new-born animals which manifested no signs of dyspnea when exposed to dander in the inhalation chamber. These animals were injected intravenously with horse dander solution and manifested no reaction after the injection. This indicates that only specifically sensitized new-born animals will evidence anaphylactic symptoms. The adult guinea pigs have been shown to be non-sensitive because none of them manifested any signs of dyspnea during the period of sensitization.

In Family 1 we have an animal sensitized by inhalation of dry horse dander before pregnancy which, at various times throughout her pregnancy, was brought into contact with this dust and manifested severe respiratory anaphylaxis (asthma). When her offspring were 5 days old and were given an intravenous injection of dander extract, they died in acute anaphylactic shock. These 2 offspring demonstrate that an animal sensitized merely by inhalation and which has had

TABLE 1.
Summary of Protocols.

	Family	Mother			Offspring		
		Respiratory anaphylaxis before birth of offspring	Respiratory anaphylaxis after birth of offspring	Intravenous injection	No.	Respiratory anaphylaxis	Intravenous injection
1	1030	++	Not done	+++	1454 1455	Not done Not done	+++ +++
2	1311	Not done ++	Not done ++	Not done ++	1344 1516 1517 1518	+ ++ ++ ++	Not done +++ +++ +++
3	1509	++	+	++	1594 1595	++ ++	+++ +++
4	1476	++	-	++	1606	++	+++
5	1410	++	+	++	1489 1490	++ ++	+++ +++
6	1217	++	+	++	1626 1627	++ ++	++ +++
7	1466	-	-	+ -	1512 1513	++ ++	+++ ++
8	1467	++	+	+	1583 1584 1585	+ + +	+ + +
9	1215	++	++	++	1580 1581 1582	++ ++ ++	++ ++ ++
10	1481	++	+	+	1631 1632	+ +	++ ++
11	1480	++	-	+	1623 1624 1625	+ + +	+ + +
12	1479	Not done	+	++	1496 1497	+ +	+ +

- No reaction.

+ - Slight symptoms of anaphylaxis.

+ Moderate symptoms of anaphylaxis.

++ Marked symptoms of anaphylaxis.

+++ Typical anaphylactic death with distended lungs.

TABLE I—*Concluded.*

	Family	Mother			Offspring		
		Respiratory anaphylaxis before birth of offspring	Respiratory anaphylaxis after birth of offspring	Intravenous injection	No.	Respiratory anaphylaxis	Intravenous injection
13	1460	Not done	—	++	1482	++	+++
					1483	—	+
14	1265	Not done	Not done	+++	1308	Not done	+-
					1309	Not done	+-
15	1173	++	—	—	1541	+	++
16	1461	Not done	++	++	1493	—	—
					1494	—	+
					1495	—	+
17	1408	+	+-	+-	1596	+-	+-
					1597	+-	+-
					1598	+-	+-
					1599	+-	+-
18	1463	+	—	—	1586	+-	—
					1587	+-	—
					1588	+-	—
19	1469	Not done	—	—	1491	—	—
					1492	—	—
20	1310	Not done	—	Not done	1336	—	Not done
21	1266	Not done	++	Not done	1335	—	Not done
22	1475	Not done	—	—	1484	—	—
					1485	—	—
23	1473	—	—	—	1494	—	—
					1495	—	—
					—	—	—
24	1333	—	—	Not done	1452	Not done	—
					1453	Not done	—
25	1468	—	—	—	1514	—	—
					1515	+	—
26	1270	Not done	Not done	—	1334	—	—

respiratory anaphylaxis during pregnancy can give birth to offspring which are sensitive to the same substance.

Families 2, 3, 4 and 5 demonstrate the transmission of respiratory anaphylaxis from mother to offspring, that is, the offspring, when exposed to horse dander for the first time gave evidence of respiratory anaphylaxis.

Families 6, 7, 13 and 16 also demonstrate the transmission of respiratory anaphylaxis from a mother to her offspring but show in all instances a varying degree of sensitization in the offspring. In 3 families 1 offspring died in anaphylactic shock while the other offspring showed anaphylaxis with recovery. In Family 16, 1 offspring was entirely negative while the other 2 showed moderate anaphylaxis. In this last case the mother was strongly anaphylactic.

In Families 8, 9, 10, 11 and 12 we have instances of respiratory anaphylaxis to a more moderate degree in both mother and offspring than has been evidenced in the above families.

Family 13 demonstrates active sensitization of a fetus in utero.⁵ The mother was exposed to the horse dander dust for 1 hour only. 2 days after her initial contact she gave birth to 2 offspring. These offspring were permitted to live in a normal environment for 27 days and were then exposed to dander in the inhalation chamber for the first time. They showed marked respiratory anaphylaxis (asthma) in the inhalation chamber and 1 died when given an intravenous injection of dander extract. Here we have an instance of the transfer not of antibodies from a mother which has suffered from respiratory anaphylaxis during pregnancy, but the passage of antigen through the upper respiratory tract into the mother's circulation and thence into the circulation of the fetus. In the fetal blood this antigen brings about the development of active sensitization after a suitable incubation period has elapsed.

In Families 14 and 16 we have instances in which the mother was profoundly sensitive and the offspring were only moderately sensitive. The only symptoms of anaphylaxis shown in Family 14 was that of suffusion of both eyes of 1 offspring. The symptoms of suffusion were also shown by Animals 1509 and 1479.⁶

⁵ Ratner, B., Jackson, H. C., and Gruehl, H. L., *J. Immunol.*, 1927, xiv, 303.

⁶ Ratner, B., Jackson, H. C., and Gruehl, H. L., *Proc. Soc. Exper. Biol. & Med.* 1927, xxiv, 444.

Families 7 and 15 show interesting instances of the sensitization apparently having worn off in the mother whereas there is still a moderate anaphylactic sensitization present in the offspring.

In Families 17 and 18 we have only an extremely moderate degree of respiratory anaphylaxis in the mother and in the offspring. In Family 17 where the sensitization was carried on over a long period of time the transmission of respiratory anaphylaxis was no more marked than in Family 18.

In Families 19, 20, 21 and 22 there has been an absence of transmission of respiratory anaphylaxis probably because of the short period of exposure of the mother. In these cases the mothers themselves were not sensitive.

In Families 23, 24, 25 and 26, on the other hand, we have instances of a sufficiently long period of sensitization in the mother with neither the mothers nor the offspring showing any sensitization.

In Family 2 we have an instance of two litters born to the same animal. In the case of the first litter the time between the beginning of sensitization and confinement of the mother was only 23 days and the offspring showed only moderate anaphylaxis. In that of the second litter, born after the mother had had many attacks of respiratory anaphylaxis (asthma), throughout her pregnancy, the offspring were profoundly sensitive and all died after an intravenous injection of dander extract.

DISCUSSION.

Normal guinea pigs, whether they be pregnant mothers or offspring only a few days old, will not manifest dyspnea when exposed to a dust to which they are not sensitive. This has been demonstrated by our many experiments.

In order to prove the anaphylactic character of the symptoms and to rule out pneumonia or any other pathologic condition of the guinea pig which might simulate the dyspnea found in respiratory anaphylaxis,⁷ the animals received an intravenous injection of an extract of the dust on the same day that they demonstrated this syndrome and in all cases necropsies were performed.

⁷ Ratner, B., Jackson, H. C., and Gruehl, H. L., *Am. J. Dis. Child.*, 1927, xxxiv, 23.

It may be of interest to comment on the fact that in 26 families studied we have no single instance of miscarriage during the attacks of respiratory anaphylaxis (asthma) even in the latter period of pregnancy.

Analysis of our protocols demonstrates that a mother guinea pig which has manifested respiratory anaphylaxis during pregnancy may passively³ transmit the state of hypersensitivity to her offspring in utero. In one instance this was brought about by an active sensitization in utero. When the offspring are first brought into contact with a dust to which the mother is sensitive, they exhibit respiratory anaphylaxis (asthma) of the same sort she showed.

There were certain variations in the transmission which call for further discussion.

Because of the nature of the experiments, it has been impossible to sensitize and expose these mothers in a uniform manner. For this reason—as is evident in Table I—instances occurred in which sensitization was firmly established in the mothers and was transmitted, other instances in which the mothers were more profoundly sensitized than the offspring or conversely in which the offspring had a higher degree of sensitization than the mother; instances in which the sensitization established was of a moderate grade; and finally instances in which there was no sensitization of either mother or offspring. Several of the families in this last group undoubtedly had too short a period of exposure for the establishment of sensitization, while others, although the period was long enough apparently, were not sensitized.

In Family 2 we have an interesting example of the influence of the period upon the results. The offspring of the first litter was only moderately sensitive whereas those of the second litter, after the mother had shown profound respiratory anaphylaxis throughout her pregnancy, were sensitive and died in typical anaphylactic shock.

We were fortunate in having 1 family in which the mother was exposed for only 2 days before confinement. The offspring in this instance could not have received sensitizing antibodies but must have developed an active sensitization from antigen transmitted in utero. This mechanism has been demonstrated by us in a previous study.

³ Ratner, B., Jackson, H. C., and Gruehl, H. L., *J. Immunol.*, 1977. xiv, 291.

In our previous work⁹ it was shown that guinea pigs injected prior to or during pregnancy might give birth to sensitive offspring; and in another study¹⁰ we presented instances of human mothers eating excessively of certain foods during pregnancy who gave birth to children sensitive to those foods.

We believe the experiments presented in this paper demonstrate a manner in which the human offspring of an asthmatic mother might be sensitized. Furthermore, the experiments indicate that mothers with the asthma of anaphylaxis may not always give birth to asthmatic children and on the other hand that mothers, themselves not asthmatic, may transmit sensitizing antigen to their offspring.

CONCLUSIONS.

1. A further method is offered whereby sensitization in utero may be established.
2. Respiratory anaphylaxis—induced in a pregnant guinea pig by the inhalation of a dry antigenic dust—can thus be transmitted from mother to offspring.
3. A guinea pig thus sensitized in utero, when brought into contact for the first time with an anaphylactogenic dust to which the mother was sensitized, will manifest respiratory anaphylaxis.
4. The transmission of this hypersensitiveness may be brought about passively through the transmission of sensitizing antibodies.
5. A fetus may be actively sensitized in utero by a mother which has inhaled the antigenic dust and has not herself been sensitive at the time of birth.
6. This state of hypersensitiveness may be transmitted in varying degrees of intensity and when 2 or more offspring are born in the same litter, they may, in some instances, be sensitized to an equal degree and sometimes to different degrees.
7. This state of hypersensitiveness can be transmitted through more than 1 litter.
8. All animals cannot be made hypersensitive.

⁹ Ratner, B., Jackson, H. C., and Gruehl, H. L., *J. Immunol.*, 1927, xiv, 249, 267, 275, 291, 303.

¹⁰ Ratner, B., *Am. J. Dis. Child.*, 1928, xxxvi, 277.



A "SOLUBLE SPECIFIC SUBSTANCE" DERIVED FROM GUM ARABIC.*

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The unforeseen identification of the so-called "soluble specific substances" (1) of *Pneumococcus* as polysaccharides (2) has led to an accumulation of evidence that analogous sugar derivatives play an important part in the immunological relationships of micro-organisms of the most diverse types (3). Thus, specifically reacting substances with the properties of carbohydrates have also been isolated from the Friedländer bacillus, the tubercle bacillus, the typhoid-colon group, and yeast; and evidence has been obtained of the existence of similar substances in *Streptococcus*, the anthrax bacillus, and other pathogenic microbes. This wide distribution of specifically reacting polysaccharides made it seem not improbable that there might occur among higher plant forms other sugar derivatives with specific properties. A number of water-soluble gums of plant origin were therefore tested against anti-pneumococcus sera of Types I, II, and III, and of these gums several were found to give the precipitin reaction. Since occasional samples of gum arabic (gum acacia) precipitated Type II (and Type III) antiserum at as high a dilution as 1:25,000 (*cf.* Table I, 47₂), but did not precipitate normal horse serum or Type I antiserum, this gum was chosen for further study.

It was soon found that the ordinary methods of fractional precipitation from neutral, acid, or alkaline solutions yielded products differing

* An abstract of this paper was presented at the Annual Meeting of the American Chemical Society in Philadelphia, in Sept., 1926. The paper itself was submitted for publication at the same time, but was withdrawn until more positive analytical data on the calcium aldobionate were available. In the meantime Cretcher and Butler have also published a note (*Science*, Aug. 3, 1928) indicating the presence of an aldobionic acid among the products of hydrolysis of gum arabic and have a paper in press giving further details.

little in their specific reactivity. Since it was known that the soluble specific substance of *Pneumococcus* was comparatively resistant to strong acid in the cold (2*b*, p. 305), fractional hydrolysis of this type was resorted to in the hope that the non-specific portions of the gum would prove the more easily hydrolyzed. This appeared to be the case, as the polysaccharide fraction recovered was found to possess a degree of specificity comparable with that of the bacterial specific substances (*cf.* Table I, 47_{10BIV}, 56). From the fact that 50 per cent of material 100 to 150 times as active as the original gum was recovered it is evident, however, that the process involves more than a mere hydrolysis of accompanying inert material. This point will be taken up more fully in the discussion.

The gum arabic purified in this way resembled in its physical properties the arabic acid so frequently described as the principal constituent of the gum (4). On hydrolysis, however, it yielded mainly galactose and an acid fraction consisting of at least 2 substances. Investigation of these acids has indicated that they are possibly disaccharide (aldobionic) acids of the type recently described as the principal product of hydrolysis of the soluble specific substance of Type III pneumococcus (3*a* and 3*b*). Such a relationship would be of considerable theoretical interest in a study of the chemistry of bacterial specificity. The large quantities of starting material available should facilitate the investigation of aldobionic acids to an extent impossible in the case of the polysaccharides elaborated by pathogenic bacteria.

EXPERIMENTAL.

1. Isolation of Reactive Material from the Original Gum.

200 gm. of Squibb's powdered gum acacia were dusted slowly into 1 liter of chilled 1:1 hydrochloric acid. The mixture was stirred until solution was complete and allowed to stand at room temperature for 2 days. A small amount of dark, insoluble material was centrifuged off and the clear liquid precipitated with about 3 volumes of chilled alcohol. After the gum had settled the supernatant was poured off and the precipitate macerated with fresh alcohol. After several hours this was decanted and the gum was dissolved in water, centrifuged if necessary, and reprecipitated with redistilled acetone in the cold. After several hours the precipitate was stirred with fresh acetone, ground up under acetone when thoroughly hardened, filtered, washed with acetone until free from hydrochloric acid, and dried *in vacuo* over calcium chloride and crushed sodium hydroxide.

The yield was 70 to 80 gm. This product (Table I, 56) corresponded closely to that obtained in 97.5 gm. yield by a single acid treatment of 24 hours (47₁₀) and to preparation 47_{10BIV} obtained in 64 gm. yield by 2 single acid treatments of 24 hours each. It was sometimes necessary to redissolve the gum in water a second time and reprecipitate with acetone in order to remove all chlorine ion.

The product so obtained still contained about 0.3 per cent of nitrogen, or the entire amount in the original gum. 60 gm. of preparation 47_{10BIV} were therefore dissolved in 300 cc. of water and stirred $\frac{1}{2}$ hour with 5 cc. of 30 per cent sodium nitrite solution and 25 cc. of acetic acid. About 1.5 volumes of acetic acid were then added, precipitating most of the gum. After 2 hours the deposit was drained, dissolved in about 200 cc. of water, and reprecipitated with acetic acid. It was finally treated with successive portions of redistilled alcohol and acetone, ground up, washed thoroughly with acetone, and dried as before. The yield was 42.3 gm. This product (Table I, 51E) contained less than 0.1 per cent of nitrogen and reacted with Type II anti-pneumococcus serum at a dilution of 1:5,000,000. It also precipitated Type III anti-pneumococcus serum. The fraction of the gum not thrown down by the acetic acid resembled the precipitated portion in all its properties, but it contained 0.5 per cent of nitrogen and was somewhat less reactive with Type II serum.

The purified gum is a white powder, readily soluble in water. It possesses marked acidic properties and rotates the plane of polarized light weakly to the left, somewhat more strongly on neutralization. It gives a positive naphthoresorcin test. When hydrolyzed it yields 68 per cent of reducing sugars, calculated as glucose, but since about one-third of the products of hydrolysis appear to be disaccharide or polysaccharide acids (see below), the actual yield of reducing sugars is higher. The pentose content, 19 per cent, calculated from the yield of furfural on distillation with hydrochloric acid,¹ is about one-half that of the original gum, so that much of the portion hydrolyzed in the method of preparation consisted of pentose or pentosan. Part of the remaining material which reacts as pentose is accounted for by the sugar acid fraction. The principal hexose component of the purified gum is galactose.

¹ A modification of Pervier and Gortner's method (5) was used. Instead of titrating at an acidity of 4 per cent and plotting the end-point with the aid of a bromine electrode, galvanometer, and stop-watch, it was found simpler to use an outside spot indicator of starch iodide solution. At an acidity of 3 per cent, the end-point is taken as the first burette reading at which a spot test is still obtained after 2 minutes. Large drops should be withdrawn for the test.

2. Attempts at Further Purification of the Specific Fraction.

The specific fraction of the gum is incompletely precipitated by barium hydroxide in large excess. The precipitate soon turns yellow, and as will be seen from Table I the recovered gum (51B) shows practically the same properties as the original material.

Uranyl nitrate also precipitates the gum incompletely when the excess acid is neutralized, but in this case also no purification results.

Partial adsorption of specific gum on "Type C" aluminium hydroxide (6) resulted only in a product with the properties of the starting material (Table I, 54D).

Fractionation of the specific product with hydrochloric acid and alcohol gave 3 portions with practically identical properties.

3. Precipitation of the Specific Gum by Means of Type II *Pneumococcus* Antibodies.

An attempt was made to determine whether the specific gum could be precipitated by Type II pneumococcus antiserum and recovered from the precipitate. This was of importance not only in establishing the polysaccharide as actually analogous or not to the specific polysaccharides of bacterial origin, but also in determining whether the reaction with Type III antiserum was caused by an accompanying impurity or was an inherent property of the specific polysaccharide itself. Thus, an accompanying substance which did not precipitate Type II serum, but yielded a precipitate with Type III serum, should be largely eliminated in effecting a specific precipitation with Type II serum.

3 liters of Type II pneumococcus antibody solution, prepared essentially by Felton's method (7), were precipitated by a slight excess of neutralized 1:1000 saline solution of preparation 47₁₀. The amount of precipitate was small, and only 0.07 gm. of specific gum was recovered by the method already described in detail in the case of the soluble specific substance of Type II pneumococcus (2c, p. 737).

Except for an unavoidably high nitrogen content (0.6 per cent) and a higher specific reactivity toward both sera the product (Table I, 47₁₀A) resembled the starting material in its analytical and physical properties. It still gave a precipitate with barium hydroxide in excess, gave the brown red color characteristic of galactans with orcin

TABLE I.

Properties of Gum Arabic and Derived Specific Fractions.

Preparation No.	[α] _D		Acid equivalent	Nitrogen	Reducing sugars on hydrolysis		Highest dilution precipitating pneumococcus antiserum		Ash	
	Free acid	Na salt			Total	Pentose	Type II	Type III		
47 ₂		-31.0°		0.35	77.4	40	25,000	25,000	1.4	Squibb's gum acacia
47 ₁₀	-7.5°	-8.8°	1006			15	3,000,000*	1,000,000	0.3	Single acid treatment, 24 hours
47 ₁₀ - BV	-12.0°	-16.0°	906			19	4,000,000	+	0.03	Two acid treatments
47 ₁₀ A		-20.0°	665	0.6	67.3		8,000,000	2,000,000	0.3	Recovered from immune precipitate
51B	-12.0°			0.15			4,000,000	+	0.1	From Ba(OH) ₂ precipitate
51E	-10.7°	-14.3°	856	0.08	68.0	18	5,000,000	+	0.06	47 ₁₀ AV, treated with HNO ₃ and precipitated with acetic acid
54D	-11.5°	-16.6°	723	0.2	68.5	18	4,000,000	+	0.03	Adsorbed on Al(OH) ₃
56	-7.5°	-12.5°	866	0.33	68.0	19	3,000,000†	+	0.13	Single acid treatment, 48 hours

* There is apparently relatively little antibody to the specific gum in Type II and Type III antisera. In the former case the precipitate is a transparent jelly, and the tubes containing the higher dilutions must be centrifuged in order to render the deposit more compact and more easily visible. The Type III precipitate is loose and flocculent until centrifuged.

† Highest dilution tested.

and hydrochloric acid, and showed a positive naphthoresorcin test. Not only did it react at a dilution of 1:8,000,000 with Type II anti-pneumococcus serum, but also at a dilution of 1:2,000,000 with Type

III serum, indicating that it is actually the same substance which precipitates with both sera.

While this experiment seemed fairly conclusive in establishing the purified gum as a true "soluble specific substance," it remained possible that the polysaccharide reacted with some other constituent of the serum than the pneumococcus antibodies themselves.

Accordingly a portion of the Type II antiserum was absorbed by means of a saline suspension of heat-killed Type II pneumococci. Another portion of the serum was treated with small amounts of a 1:10,000 solution of the Type II soluble

TABLE II.

Dilution of preparation 4710BY	Type II serum unabsorbed	Type II serum absorbed with Pneumococcus II	Type II serum absorbed with soluble specific substance II	Type II antibody solution absorbed with specific gum arabic
1:1,000	++++	—	—	
1:5,000	+++	—	—	
1:25,000	+++	—	—	
1:250,000	++	—	—	
1:1,000,000	—	—	—	
Saline	—	—	—	
Dilution of Type II soluble specific substance,				
1:20,000.....			—	++++
Agglutination of Type II pneumococci.....			—	++++

Dilution of serum, 2:3. Tubes were not centrifuged. After centrifugation the 1:1,000,000 dilution reacted + in the unabsorbed serum and all the tubes containing serum absorbed by *Pneumococcus II* contained a trace of scaly precipitate. Otherwise the results were unchanged.

specific substance until no further precipitate could be obtained after 2 hours at 37° and standing over night in the ice-box. The reaction of the purified gum with these sera and untreated Type II serum was then tested, giving the results shown in Table II.

4. Hydrolysis of the Specific Polysaccharide.

A. 41 gm. of preparation 51E were dissolved in water, treated with 85 cc. of concentrated sulfuric acid, diluted to 3 liters, and boiled for 4 hours, a preliminary experiment having shown a maximum reducing power after this time. The sulfuric acid was removed quantitatively with barium hydroxide, and to the filtrate, concentrated *in vacuo* to about 300 cc., basic lead acetate solution was

added in slight excess. The precipitate was suspended in water and treated with acetic acid in small amounts until only a small amount of yellow precipitate remained. The filtrate from this was again precipitated with an excess of basic lead acetate. The lead salt was decomposed with hydrogen sulfide and the solution concentrated repeatedly *in vacuo* to a syrup in order to eliminate acetic acid. The residue was taken up in hot water, boiled with acid-washed Norite, and concentrated to dryness, finally in a high vacuum. The yield of crude sugar acid was 5.5 gm. A determination of the reducing power by the Shaffer-Hartmann micro-method (8) gave a value of 44.6 per cent, calculated as glucose.

0.3329 gm., made up to 15 cc. with H_2O : α_D , 0.39° , $l = 2$. $[\alpha]_D = +8.8^\circ$.

1 cc. of the same solution required 2.62 cc. 0.02 N NaOH for neutralization to phenolphthalein. Acid equivalent, 427. Calculated for $C_{12}H_{20}O_{12}$, 356.

The filtrates from the first and second precipitations of the lead salt were freed from lead, concentrated to small bulk, boiled with Norite, concentrated to a syrup, and, while still warm, were treated with about 2 volumes of glacial acetic acid and seeded with a few crystals of galactose. Crystallization took place rapidly, and the solid cake which formed over night in the ice-box was crushed, sucked off *in vacuo* on a Buchner funnel, and washed first with chilled 66 per cent acetic acid, then with the glacial acid, and finally with alcohol. The yield was 8.3 gm., with an initial $[\alpha]_D$ of $+120.5^\circ$.

1 gm. of the crude sugar, oxidized with warm 1:1 nitric acid, began to deposit crystals within a few hours and ultimately yielded 0.46 gm. of mucic acid, melting at $214-215^\circ$ with decomposition.

0.1009 gm. gave 0.1279 gm. CO_2 and 0.0458 gm. H_2O .

Calculated for $C_6H_{10}O_6$: C, 34.27 per cent; H, 4.80 per cent. Found: C, 34.57 per cent; H, 5.08 per cent.

6.8 gm. of the sugar itself were dissolved in water, boiled with Norite, and recrystallized as before, yielding 5.4 gm. of purified sugar, which from its analysis, rotation, and the isolation of mucic acid in good yield from the crude product, was chiefly galactose.

0.1007 gm. gave 0.1480 gm. CO_2 , and 0.0620 gm. H_2O .

Calculated for $C_6H_{12}O_6$: C, 39.98 per cent; H, 6.72 per cent. Found: C 40.08 per cent; H, 6.89 per cent.

0.5513 gm., made up to 10 cc. with H_2O , gave an initial reading of 6.55° and a final value of 4.05° , $l = 1$. $[\alpha]_D$, initial, $+118.8^\circ$; final, $+73.5^\circ$.

The galactose isolated in this crop was not as pure as that recovered in later fractions (see below), but whether the impurity was the sugar acid still present in the mother-liquors, or some other sugar, has not been determined.

The filtrate from the initial crop of galactose was repeatedly diluted with water and concentrated *in vacuo* in order to remove acetic acid. It was then diluted to about 350 cc. and again treated with basic lead acetate solution, yielding a heavy

precipitate. This salt was worked up as was the first lead salt, and yielded 3.9 gm. of a crude sugar acid resembling the first product except in its rotation and somewhat lower reducing value, the latter being 39.2 per cent, calculated as glucose.

0.3064 gm., made up to 15 cc. with H_2O : α_D -0.17° , $l = 2$. $[\alpha]_D = -4.2^\circ$.

2 cc. of the same solution required 4.53 cc. 0.02 *N* NaOH for neutralization to phenol red. Acid equivalent, 450. Calculated for $C_{12}H_{20}O_{12}$, 356.

The filtrate from the above lead salt was freed from lead and acetic acid, taken up in a little water, neutralized with barium hydroxide, and again concentrated to a thick syrup. This was boiled with 3 successive 200 cc. portions of 90 per cent alcohol. The insoluble residue, purified over the lead salt, gave 2.0 gm. of a product which appeared to be a mixture of sugar and sugar acid, but was not further investigated. The alcoholic solutions after concentration to a syrup readily yielded 3.3 gm. of galactose, which melted at $161-163^\circ$ after recrystallization.

0.5522 gm., made up to 10 cc. with H_2O , gave an initial reading of 7.52° and a final value, after addition of 0.5 cc. concentrated aqueous NH_3 , of 4.11° , $l = 1$. $[\alpha]_D$, initial, $+136.2^\circ$; final, $+78.2^\circ$.

In Beilstein, 3rd edition, vol. i, p. 911, the rotations given for pure galactose are $[\alpha]_D^{20} +140^\circ$, 80.5° , respectively.

The mother liquors from the galactose contained but 1.7 gm. of sugar, calculated as glucose, and were not investigated.

(B).² 49 gm. of a product (active with Type II serum at a dilution of 1:4,000,000) were hydrolyzed as in the previous instance. After removal of the sulfuric acid the concentrated solution was boiled with calcium carbonate and Norite, filtered, concentrated to small bulk, and fractionated with methyl alcohol as in the case of the calcium aldobionate derived from the Type III pneumococcus (3b). The partially purified salt thus obtained was further fractionated into three arbitrary portions with the aid of methyl alcohol and acetone.

Fractions.....	I per cent	II per cent	III per cent	Theory per cent
Calcium.....	5.8	6.7	5.5	5.3
Reducing sugars (as glucose) (Schaffer-Hartmann).....	44.8	44.3	34.4	48.0
Aldose (as glucose) (Willstätter-Schudel)....	53.3	58.1	61.5	48.0

Fraction I³ thus corresponds fairly closely to a calcium aldobionate, $(C_{12}H_{19}O_{12})_2Ca$, while the succeeding fractions show increasing contamination.

² The experimental work in this section was carried out by one of us (M. H.) in the laboratories of the Mt. Sinai Hospital and the Presbyterian Hospital, New York. For the facilities offered by these institutions, and for the kindness of Dr. Forrest E. Kendall, of the Presbyterian Hospital, in carrying out the analyses, the writers wish to express their hearty thanks.

³ A crystalline cinchonidine salt, and through this, the crystalline aldobionic acid have since been isolated, and will form the subject of a separate communication.

The analyses indicate that the chief impurity in Fraction II is possibly a salt of the type of calcium glucuronate, while Fraction III presumably contains galactose, especially as the mother-liquors from this deposited crystals of galactose on standing (melting point, 160-168°; yielded mucic acid, melting point, 206-207°, on oxidation).

DISCUSSION.

That a polysaccharide with specific properties should occur among the higher plants without any apparent relation to the life processes of micro-organisms, is additional evidence of the wide-spread occurrence of carbohydrates with immunologically specific properties. Speculation as to their function and chemical and immunological relationships, while enticing, must be postponed until more information is at hand. There is evidence which has been interpreted by Beijerinck (9) as pointing to the elaboration of gum arabic as a result of the activities of molds, and by Greig Smith (10) as showing the gum to be a product of the metabolism of certain bacteria. As neither of these workers has proved, however, that contamination of the gum with the appropriate organism did not take place after its formation the hypothesis that gum arabic originates through the activities of micro-organisms, though attractive from the standpoint of bacterial specificity, must be considered as unproved.

In fact, certain chemical data obtained in the present investigation argue against this point of view. On partial hydrolysis about one-half of the original material is recovered with its specific activity increased 100 to 150-fold, showing that the specifically reacting gum does not exist as such in the original gum arabic, but is formed from it, probably by removal of a pentose grouping in glucosidic union, since the specific fraction contains less than one-half the pentose of the gum arabic itself. The low reactivity of the original gum would then be accounted for on the basis of traces of the specific material formed on exposure, in the process of refining, or by enzyme action. It is not excluded however, that the specific gum owes its presence to the synthetic activity of the strong hydrochloric acid on the hydrolytic products of the original gum (11), although this would seem less probable.

Another finding of chemical interest is the large proportion of complex sugar acids. It has recently been shown (3a and 3b) that the chief product of the hydrolysis of the soluble specific substance of

Type III pneumococcus is an aldobionic acid of which one component is glucose and the other glucuronic acid. A similar acid also forms one of the hydrolytic products of the soluble specific substance of the Type A Friedländer bacillus (12). At least one of the crude acid fractions from the hydrolysis of the specific gum arabic corresponds roughly to an aldobionic acid, while in the other the agreement is not so good. While both of these acid fractions may still be mixtures, their rough correspondence to important hydrolytic products of bacterial specific polysaccharides is of interest.

While the exact significance of these more or less complex sugar acids is still to be established, the finding of at least two of these acids as hydrolysis products of the specific gum arabic renders it evident that the specific gum, if actually a single substance in its present state of purity, is a more complex product than the bacterial specific polysaccharides hitherto investigated in detail. The recovery of the specific gum from its precipitate with Type II antipneumococcus serum with its reactivity for both Type II and Type III sera augmented (see experimental part) may be taken as evidence that it is a single constituent of the gum which precipitates both sera. This is in agreement with the relative complexity of the specific gum, since a substance containing the molecular groupings necessary for reaction with antibodies to both Type II and Type III pneumococci might be expected to yield more varied products on hydrolysis than one precipitating either serum alone. Whether any of these hydrolysis products of the specific gum arabic and the specific polysaccharides of Type II and Type III pneumococci are identical cannot be stated as yet. Thus far galactose has been found only in the first of the three, and this constitutes a marked difference.

From Table II it will be seen that Type II antipneumococcus serum which has been precipitated by the specific gum arabic still retains practically unimpaired its agglutinating power for the Type II pneumococcus and its precipitating power for the Type II soluble specific substance, whereas all of the antibodies are removed on absorption of Type II antiserum with Type II pneumococcus. This lack of reciprocal antibody absorption is suggestive of the relationship between *Pneumococcus* Type II and Type B Friedländer bacillus, in which the writers found (13) a certain chemical similarity between the specific polysaccharides of these bacteria and a corresponding immunological

relationship between the micro-organisms themselves. The serological and antigenic similarity of the otherwise unrelated bacteria was interpreted as an example of heterogenetic specificity; it is not unlikely that the reactions of specific gum arabic with Types II and III antipneumococcus sera may be accounted for in the same way.

SUMMARY.

1. By partial acid hydrolysis a specific carbohydrate may be isolated from gum arabic (gum acacia). This carbohydrate is comparable in its precipitating activity for Type II (and Type III) antipneumococcus serum with the bacterial soluble specific substances themselves.

2. On hydrolysis this fraction yields galactose and two or more complex sugar acids, one of which appears to be a disaccharide acid comparable with those isolated from the specific polysaccharides of the Type III pneumococcus and the Type A Friedländer bacillus.

3. The significance of these findings is discussed.

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BIOMETRY OF CALCIUM, INORGANIC PHOSPHORUS, CHOLESTEROL, AND LIPOID PHOSPHORUS IN THE BLOOD OF RABBITS.

III. INFLUENCE OF VARIOUS TYPES OF LIGHT ENVIRONMENT.

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In previous papers (1, 2) the calcium, inorganic phosphorus, cholesterol, and lipid phosphorus found in the blood of rabbits as they were received from the dealer were compared with results obtained for animals living under laboratory conditions. The difference in mean values, in the variations that occurred, in the trends of the curves, and in the chemical relationship seemed to warrant the conclusion that all of these substances were affected by light. Since it had been found that calcium and inorganic phosphorus are affected by various types of light environment (3), it seemed desirable to extend the observations which had been made for the purpose of determining whether cholesterol and lipid phosphorus were also affected by light and to study the action of a wider range of light conditions. The comparisons were made with complete exclusion of light, continuous exposure to light supplied by Cooper Hewitt lamps, exposure to diffuse filtered sunlight of variable intensity, and exclusion of light interrupted by brief periods of exposure to ultra-violet light. The results of these experiments will be reported in the present paper.

Materials and Method.

In the experiments presented, 3 animal rooms with similar environmental conditions other than those of lighting were employed. One group of 10 animals which may be designated as the open laboratory group, were kept in a well lighted (sunlight), well ventilated room with a southern exposure.

Two groups of 10 animals each were kept in a room from which all light was excluded. Other living conditions were the same as those of animals living in the open laboratory. During the course of the experiment, beginning on Feb. 1, 1928,

one group of animals was exposed for an hour each day to the unfiltered radiations of a quartz-mercury arc lamp (80 volts, 4.2 amperes) at a distance of 4.5 meters. These animals shall be designated as the ultra-violet group. The other animals which remained in the dark room throughout the experiment shall be designated as the dark group. Both of these groups of animals received some light from two other sources. For a brief period each day a 30 watt Mazda lamp was used in the dark room for cleaning cages, feeding, and making certain necessary observations. The animals were also exposed to diffuse filtered sunlight for a brief period once a week when they were brought into the laboratory for bleeding purposes.

The fourth group of animals which shall be designated as the light group was kept in a room similar to the others with sunlight excluded. In this room a *constant* source of light was furnished by 13 Cooper Hewitt low pressure mercury arcs, Type P, in crown glass arranged in 3 superimposed rows on an iron frame placed in the center of the room. The animals in individual cages were placed in racks on either side of and parallel with the mercury arcs at a distance of 1 meter. The average intensity of the light was calculated to be 2000 foot candles.

The spectrogram of this type of mercury arc in crown glass shows that the range of light rays is from 3022 to 5790 Ångström units with the majority falling in the 3650 and 5790 portion of the spectrum. The exposure to the Cooper Hewitt mercury arcs was begun on Jan. 20, 1928.

The doors of the cages were opened to permit free access of light but no depilatory measures were used for any group of animals, and at no time throughout the experiment could there be demonstrated evidence of dermatitis, conjunctivitis, or any other inflammatory process which might be attributed to the action of either ultra-violet or Cooper Hewitt radiation.

The temperature of the 3 rooms was satisfactorily maintained at 70° to 75°F. and the humidity of each varied with that of the outside atmosphere.

The animals selected for this experiment were all male rabbits of mixed grey and brown breeds from 6 to 8 months old. They were sexually mature but had not yet attained their full growth. All animals were caged separately and fed a uniform diet of hay, oats, and cabbage.

The data presented in this paper are derived from experiments, the procedures of which were described in detail in a preceding paper (1).

The determinations of calcium and inorganic phosphorus of blood serum, cholesterol and lipid phosphorus of the whole blood were made on the same sample of blood. Determinations were made, beginning on Jan. 5, 1928, at weekly or biweekly intervals until May 31, 1928, resulting in 15 recorded observations for the light, dark, and open laboratory groups; 14 observations were made on the ultra-violet group.

In all text-figures and tables the lipid phosphorus is calculated and presented as lecithin.

RESULTS.

The results of the observations made in this experiment are presented in the form of tabulated summaries, Tables I to VI, which are supplemented by a series of graphs, Text-figs. 1 to 14. The mean values contained in Tables I to VI inclusive have been smoothed by the formula $\frac{A + 2B + C}{4}$ and are presented in the graphs in terms of per cent variations from standard mean values, using for this purpose values obtained for animals living in the open laboratory throughout the year. These results were reported in a previous paper (2) and shall serve also as a basis of comparison for certain ratio values obtained in the present experiment. The average values for this group of animals were calculated to be for calcium 15.7, inorganic phosphorus 4.65 mg. per 100 cc. of blood serum, and for cholesterol 58.2, lecithin 118.4 mg. per 100 cc. of whole blood. Text-figs. 1 to 4 inclusive represent the per cent variation of the 4 blood constituents; Text-figs. 5 to 10 inclusive, the per cent variation of the 6 possible ratios of the same 4 blood constituents; and Text-figs. 11 to 14 inclusive, the distribution of individual values for calcium, inorganic phosphorus, cholesterol, and lecithin.

TABLE I.
Calcium Values for Consecutive Determinations.

Date	Open laboratory			Dark			Cooper Hewitt			Date	Ultra-violet		
	Mean	Stand- ard devia- tion	Coeffi- cient of varia- tion	Mean	Stand- ard devia- tion	Coeffi- cient of varia- tion	Mean	Stand- ard devia- tion	Coeffi- cient of varia- tion		Mean	Stand- ard devia- tion	Coeffi- cient of varia- tion
	mg. per 100 cc.	mg.	per cent	mg. per 100 cc.	mg.	per cent	mg. per 100 cc.	mg.	per cent		mg. per 100 cc.	mg.	per cent
1928										1928			
Jan. 5	16.4 \pm 0.23	1.10	7.06	15.5 \pm 0.17	0.80	5.16	15.6 \pm 0.17	0.82	5.26	Jan. 12	14.5 \pm 0.09	0.46	3.22
" 19	15.2 \pm 0.14	0.67	4.45	15.3 \pm 0.20	0.95	6.22	15.2 \pm 0.11	0.55	3.62	" 31	15.2 \pm 0.34	1.60	10.58
" 26	15.6 \pm 0.17	0.80	5.16	16.4 \pm 0.17	0.80	4.87	16.6 \pm 0.19	0.92	5.19	Feb. 7	15.8 \pm 0.16	0.75	4.75
Feb. 2	15.3 \pm 0.10	0.48	3.15	15.3 \pm 0.10	0.48	3.16	15.7 \pm 0.15	0.72	4.58	" 14	14.6 \pm 0.08	0.38	2.60
" 9	15.4 \pm 0.16	0.75	4.88	14.9 \pm 0.14	0.65	4.42	15.2 \pm 0.10	0.51	3.36	" 21	15.4 \pm 0.20	0.95	6.17
" 16	14.8 \pm 0.11	0.52	3.52	14.6 \pm 0.11	0.53	3.64	15.1 \pm 0.12	0.58	3.90	" 28	15.7 \pm 0.22	1.05	6.72
" 23	16.4 \pm 0.32	1.50	9.14	16.9 \pm 0.33	1.59	9.51	17.0 \pm 0.35	1.65	9.70	Mar. 6	16.0 \pm 0.14	0.66	4.15
Mar. 1	15.8 \pm 0.04	0.19	12.34	16.3 \pm 0.14	0.67	4.11	16.1 \pm 0.11	0.54	3.35	" 13	15.5 \pm 0.14	0.69	4.47
" 8	15.6 \pm 0.09	0.45	8.68	15.7 \pm 0.12	0.58	3.70	15.5 \pm 0.15	0.71	4.58	" 26	15.6 \pm 0.14	0.70	4.58
" 22	14.7 \pm 0.07	0.34	23.74	15.2 \pm 0.12	0.57	3.75	14.7 \pm 0.11	0.55	3.74	" 29	15.4 \pm 0.19	0.91	5.92
" 29	14.8 \pm 0.10	0.48	3.25	15.8 \pm 0.16	0.74	4.75	15.2 \pm 0.08	0.40	2.66	Apr. 12	15.1 \pm 0.13	0.61	4.08
Apr. 12	14.8 \pm 0.13	0.62	4.15	15.0 \pm 0.12	0.57	3.82	14.9 \pm 0.16	0.61	5.35	" 26	14.6 \pm 0.16	0.75	5.16
" 26	14.9 \pm 0.13	0.62	4.20	15.1 \pm 0.20	0.94	6.27	15.0 \pm 0.14	0.69	4.64	May 10	14.8 \pm 0.32	1.53	10.53
May 10	14.8 \pm 0.13	0.63	4.29	15.9 \pm 0.20	0.96	6.09	15.2 \pm 0.20	0.96	6.36	" 31	14.2 \pm 0.30	0.96	6.78
" 31	14.9 \pm 0.14	0.64	5.10	14.7 \pm 0.14	0.67	4.44	14.7 \pm 0.13	0.63	4.29				

TABLE II.
Inorganic Phosphorus Values for Consecutive Determinations.

Inorganic Phosphorus Values for Consecutive Determinations.

Date	Open laboratory			Dark			Cooper Hewitt			Date	Ultra-violet		
	Mean	Stand-ard deviation	Coeffi-ent of varia-tion	Mean	Stand-ard deviation	Coeffi-ent of varia-tion	Mean	Stand-ard deviation	Coeffi-ent of varia-tion		Mean	Stand-ard deviation	Coeffi-ent of varia-tion
1928										1928			
Jan. 5	5.43 ± 0.10	0.48	8.87	5.64 ± 0.11	0.53	9.55	5.50 ± 0.11	0.55	10.10	Jan. 12	6.10 ± 0.16	0.78	12.85
" 19	5.26 ± 0.11	0.55	10.60	4.75 ± 0.08	0.39	8.24	4.87 ± 0.06	0.30	6.31	" 31	5.17 ± 0.10	0.49	9.61
" 26	5.40 ± 0.06	0.32	6.01	4.53 ± 0.10	0.48	10.63	5.27 ± 0.08	0.37	7.13	Feb. 7	4.99 ± 0.11	0.52	10.46
Feb. 2	5.52 ± 0.07	0.33	6.03	5.26 ± 0.06	0.27	5.30	5.05 ± 0.08	0.37	7.48	" 14	4.81 ± 0.13	0.62	12.83
" 9	5.58 ± 0.07	0.31	6.21	4.73 ± 0.05	0.26	5.68	5.01 ± 0.07	0.31	6.96	" 21	4.43 ± 0.03	0.17	3.90
" 16	5.97 ± 0.09	0.46	7.75	5.13 ± 0.09	0.45	7.91	5.41 ± 0.07	0.33	6.22	" 28	5.40 ± 0.15	0.71	13.14
" 23	5.73 ± 0.11	0.51	9.95	5.18 ± 0.16	0.79	15.36	5.26 ± 0.07	0.36	6.93	Mar. 6	5.96 ± 0.13	0.64	11.31
Mar. 1	5.18 ± 0.10	0.18	8.89	4.91 ± 0.07	0.37	7.53	5.01 ± 0.11	0.53	10.75	" 13	4.91 ± 0.09	0.43	8.76
" 8	5.15 ± 0.05	0.21	4.83	4.73 ± 0.08	0.41	8.74	5.01 ± 0.06	0.27	5.55	" 26	5.14 ± 0.08	0.39	7.65
" 22	4.78 ± 0.07	0.31	7.18	4.71 ± 0.08	0.40	8.58	4.65 ± 0.06	0.32	6.99	" 29	6.58 ± 0.20	0.91	14.29
" 29	5.05 ± 0.10	0.48	9.52	4.67 ± 0.06	0.30	6.61	5.18 ± 0.06	0.30	5.92	Apr. 12	4.99 ± 0.08	0.40	8.18
Apr. 12	4.46 ± 0.08	0.40	9.90	4.57 ± 0.07	0.33	7.35	4.62 ± 0.07	0.36	7.83	" 26	5.02 ± 0.09	0.42	8.51
" 26	4.17 ± 0.07	0.31	7.77	4.57 ± 0.07	0.31	7.48	4.77 ± 0.10	0.47	9.81	May 10	5.53 ± 0.08	0.41	7.55
May 10	4.90 ± 0.10	0.51	10.26	4.92 ± 0.09	0.43	8.81	4.92 ± 0.09	0.43	8.92	" 31	4.87 ± 0.09	0.43	8.72
" 31	4.80 ± 0.08	0.36	8.10	4.27 ± 0.08	0.40	8.61	5.07 ± 0.08	0.36	6.96				

TABLE III.
Cholesterol Values for Consecutive Determinations.

Date	Open laboratory			Dark			Cooper Hewitt			Date	Ultra-violet		
	Mean	Stand- ard devia- tion	Coeffi- cient of varia- tion	Mean	Stand- ard devia- tion	Coeffi- cient of varia- tion	Mean	Stand- ard devia- tion	Coeffi- cient of varia- tion		Mean	Stand- ard devia- tion	Coeffi- cient of varia- tion
1928	mg. per 100 cc.	mg.	per cent	mg. per 100 cc.	mg.	per cent	mg. per 100 cc.	mg.	per cent	1928	mg. per 100 cc.	mg.	per cent
Jan. 5	65.6 \pm 1.23	5.76	8.78	63.4 \pm 1.18	5.54	8.74	61.8 \pm 0.75	3.54	5.74	Jan. 12	73.3 \pm 1.35	6.36	8.68
" 19	65.2 \pm 1.20	5.66	8.68	61.8 \pm 0.92	4.32	7.00	59.3 \pm 0.77	3.63	6.12	" 31	57.5 \pm 1.23	5.76	10.03
" 26	61.9 \pm 1.39	6.56	10.59	57.0 \pm 1.18	5.56	9.75	56.1 \pm 0.82	3.88	6.82	Feb. 7	60.3 \pm 0.84	3.71	6.33
Feb. 2	61.5 \pm 1.07	5.04	8.20	60.8 \pm 1.58	7.43	12.22	57.3 \pm 1.37	6.45	11.26	" 14	65.8 \pm 0.94	4.41	6.70
" 9	61.2 \pm 1.26	5.92	9.67	58.3 \pm 1.25	5.89	10.11	55.2 \pm 0.73	3.45	6.25	" 21	71.2 \pm 2.18	10.24	14.38
" 16	61.6 \pm 1.28	6.04	9.81	58.3 \pm 1.10	5.18	8.88	62.1 \pm 0.91	4.29	5.90	" 28	66.0 \pm 1.63	7.64	11.58
" 23	60.0 \pm 1.77	8.30	13.83	58.5 \pm 0.83	3.93	6.72	55.6 \pm 1.31	6.14	11.04	Mar. 6	57.0 \pm 0.66	3.10	5.96
Mar. 1	55.2 \pm 1.36	6.39	11.57	56.5 \pm 0.69	3.22	5.69	54.2 \pm 0.82	3.83	7.06	" 13	60.9 \pm 1.20	5.63	9.24
" 8	58.7 \pm 1.47	6.89	11.73	65.8 \pm 1.54	7.20	10.94	58.0 \pm 0.80	3.74	6.45	" 26	59.1 \pm 1.15	5.40	9.14
" 22	59.5 \pm 1.46	6.84	11.49	65.7 \pm 1.32	6.20	9.43	64.4 \pm 1.11	5.19	8.05	" 29	73.4 \pm 1.10	5.15	7.02
" 29	63.9 \pm 1.91	8.96	14.02	57.7 \pm 1.00	4.70	8.14	59.7 \pm 0.95	4.90	8.21	Apr. 12	69.6 \pm 0.96	4.51	6.47
Apr. 12	65.8 \pm 2.32	10.88	16.55	61.2 \pm 0.73	3.43	5.61	60.2 \pm 0.99	4.64	7.71	" 26	68.3 \pm 1.43	6.72	9.84
" 26	71.2 \pm 2.60	12.20	17.13	64.0 \pm 1.81	8.48	13.25	66.9 \pm 1.95	9.15	13.67	May 10	80.3 \pm 1.72	8.03	10.01
May 10	64.7 \pm 1.59	7.46	11.53	67.8 \pm 1.66	7.76	11.45	61.7 \pm 1.56	7.30	11.80	" 31	67.7 \pm 1.30	5.82	8.76
" 31	66.2 \pm 1.22	5.69	8.72	59.9 \pm 0.92	3.84	6.38	83.8 \pm 1.36	6.38	10.49				

TABLE IV.
Lecithin Values for Consecutive Determinations.

Lecithin Values for Consecutive Determinations.

Date	Open laboratory				Dark				Cooper Hewitt				Date	Ultra-violet			
	Mean		Stand- ard devia- tion	Coeffi- cient of varia- tion	Mean		Stand- ard devia- tion	Coeffi- cient of varia- tion	Mean		Stand- ard devia- tion	Coeffi- cient of varia- tion		Mean		Stand- ard devia- tion	Coeffi- cient of varia- tion
	mg. per 100 cc.	mg.			per cent	mg. per 100 cc.			mg.	per cent				mg. per 100 cc.	mg.		
1923													1923				
Jan. 5	129.8	±2.97	13.11	10.10	137.9	±2.38	11.17	8.10	143.2	±0.15	7.07	4.93	Jan. 12	125.1	±1.63	7.65	6.12
" 19	145.4	±1.69	7.94	5.46	158.7	±1.46	6.86	4.32	154.8	±1.95	9.14	5.90	" 31	109.6	±1.10	5.19	4.74
" 26	112.1	±2.16	10.17	9.07	110.7	±2.60	12.19	11.01	119.2	±2.09	9.80	8.22	Feb. 7	124.6	±2.91	13.65	10.95
Feb. 2	87.7	±1.57	7.37	8.41	86.1	±1.88	8.84	10.27	96.1	±1.64	7.69	8.01	" 14	141.9	±1.90	8.92	6.29
" 9	145.3	±2.21	10.39	7.15	155.4	±2.37	11.15	7.18	151.5	±3.90	18.29	12.07	" 21	144.9	±3.51	16.48	13.73
" 16	116.8	±2.75	12.90	11.04	108.9	±4.21	19.75	18.13	121.2	±3.46	16.23	13.39	" 28	95.7	±2.24	10.53	11.00
" 23	138.4	±3.18	14.89	10.75	129.1	±6.34	29.75	23.04	115.5	±3.87	18.15	15.71	" 6	96.1	±2.43	11.39	11.85
Mar. 1	137.7	±3.55	16.66	12.10	109.2	±4.76	22.30	20.42	109.4	±4.31	20.21	18.47	" 13	134.5	±3.16	14.81	11.01
" 8	77.8	±4.02	18.85	24.22	75.1	±3.69	17.31	23.05	73.6	±2.37	11.10	15.08	" 26	96.7	±2.13	9.97	10.32
" 22	147.2	±2.34	10.95	7.43	162.5	±4.30	20.15	12.40	159.0	±4.61	21.60	13.52	" 29	111.0	±1.86	8.72	7.85
" 29	139.1	±2.71	12.70	9.13	122.3	±3.09	14.50	11.85	130.4	±3.84	18.01	13.81	" 12	126.9	±4.39	20.62	16.34
Apr. 12	124.0	±1.87	8.75	7.05	159.0	±2.09	9.82	6.17	145.3	±2.57	12.05	8.29	" 26	128.4	±3.27	15.35	11.95
" 26	129.2	±3.48	16.33	12.63	134.4	±2.65	12.45	9.26	126.6	±4.73	22.19	17.53	" 10	123.6	±3.30	15.45	12.50
May 10	129.5	±4.51	21.15	16.33	135.4	±3.73	17.48	12.91	135.0	±2.55	11.95	8.85	" 31	135.4	±1.81	10.11	9.46
" 31	119.9	±1.66	8.31	7.68	126.1	±1.44	6.21	5.37	117.1	±1.67	8.93	7.52					

TABLE V.
(a) *Coefficients of Correlation for Consecutive Group Means (Trend).*

Group	rP. Ca.	rP. Chol.	rP. Lec.	rCa. Chol.	rCa. Lec.	rChol. Lec.	N
Open laboratory.....	+0.498 \pm 0.13	-0.566 \pm 0.12	-0.097 \pm 0.18	-0.328 \pm 0.15	-0.057 \pm 0.18	+0.117 \pm 0.17	N = 15
Dark room.....	+0.021 \pm 0.21	+0.065 \pm 0.18	-0.178 \pm 0.17	-0.180 \pm 0.17	-0.256 \pm 0.16	+0.147 \pm 0.17	N = 15
Light.....	+0.491 \pm 0.13	-0.146 \pm 0.17	-0.238 \pm 0.16	-0.627 \pm 0.10	-0.619 \pm 0.10	+0.776 \pm 0.07	N = 15
Ultra-violet.....	+0.027 \pm 0.21	+0.016 \pm 0.21	-0.722 \pm 0.08	-0.575 \pm 0.12	-0.625 \pm 0.10	+0.404 \pm 0.14	N = 14

(b) *Coefficients of Correlation for Individual Mean Values.*

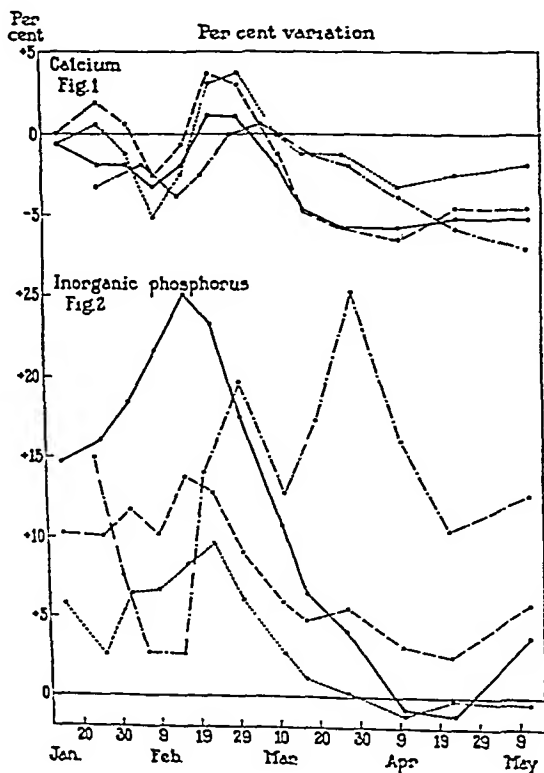
Group	rP. Ca.	rP. Chol.	rP. Lec.	rCa. Chol.	rCa. Lec.	rChol. Lec.	N
Open laboratory.....	-0.398 \pm 0.18	-0.110 \pm 0.21	-0.309 \pm 0.19	+0.034 \pm 0.22	+0.150 \pm 0.21	+0.064 \pm 0.22	N = 10
Dark room.....	-0.559 \pm 0.15	-0.008 \pm 0.22	-0.226 \pm 0.20	-0.028 \pm 0.22	+0.451 \pm 0.17	+0.061 \pm 0.22	N = 10
Light.....	-0.225 \pm 0.20	+0.331 \pm 0.19	-0.320 \pm 0.19	-0.607 \pm 0.13	-0.169 \pm 0.20	+0.403 \pm 0.18	N = 10
Ultra-violet.....	-0.258 \pm 0.20	+0.137 \pm 0.21	-0.683 \pm 0.11	+0.626 \pm 0.13	+0.646 \pm 0.12	+0.415 \pm 0.17	N = 10

(c) *Coefficients of Correlation for Entire Series.*

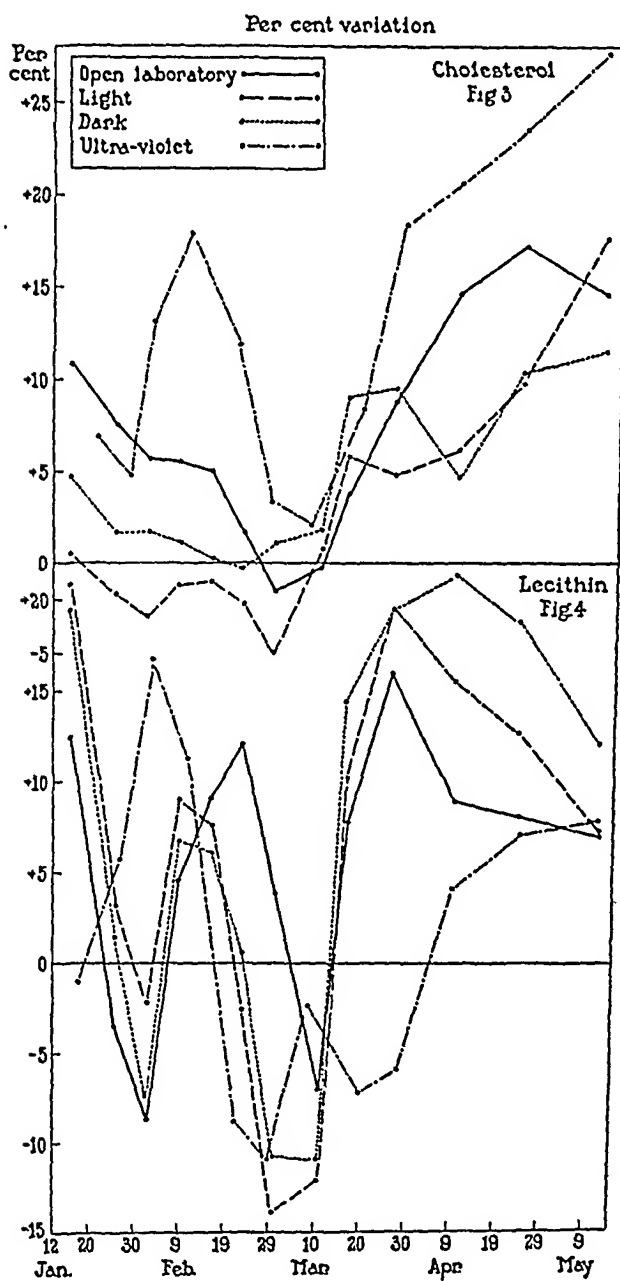
Group	rP. Ca.	rP. Chol.	rP. Lec.	rCa. Chol.	rCa. Lec.	rChol. Lec.	N
Open laboratory.....	+0.655 \pm 0.03	-0.241 \pm 0.05	-0.084 \pm 0.06	-0.069 \pm 0.06	-0.001 \pm 0.06	+0.027 \pm 0.06	N = 150
Dark room.....	-0.051 \pm 0.06	+0.045 \pm 0.06	-0.102 \pm 0.06	-0.075 \pm 0.06	-0.015 \pm 0.06	+0.169 \pm 0.05	N = 150
Light.....	+0.154 \pm 0.05	-0.052 \pm 0.06	-0.112 \pm 0.06	-0.398 \pm 0.04	-0.220 \pm 0.05	+0.113 \pm 0.05	N = 150
Ultra-violet.....	+0.031 \pm 0.05	+0.152 \pm 0.05	-0.306 \pm 0.04	+0.023 \pm 0.06	-0.115 \pm 0.06	+0.224 \pm 0.05	N = 140

TABLE VI.
Mean Values for Each Group.

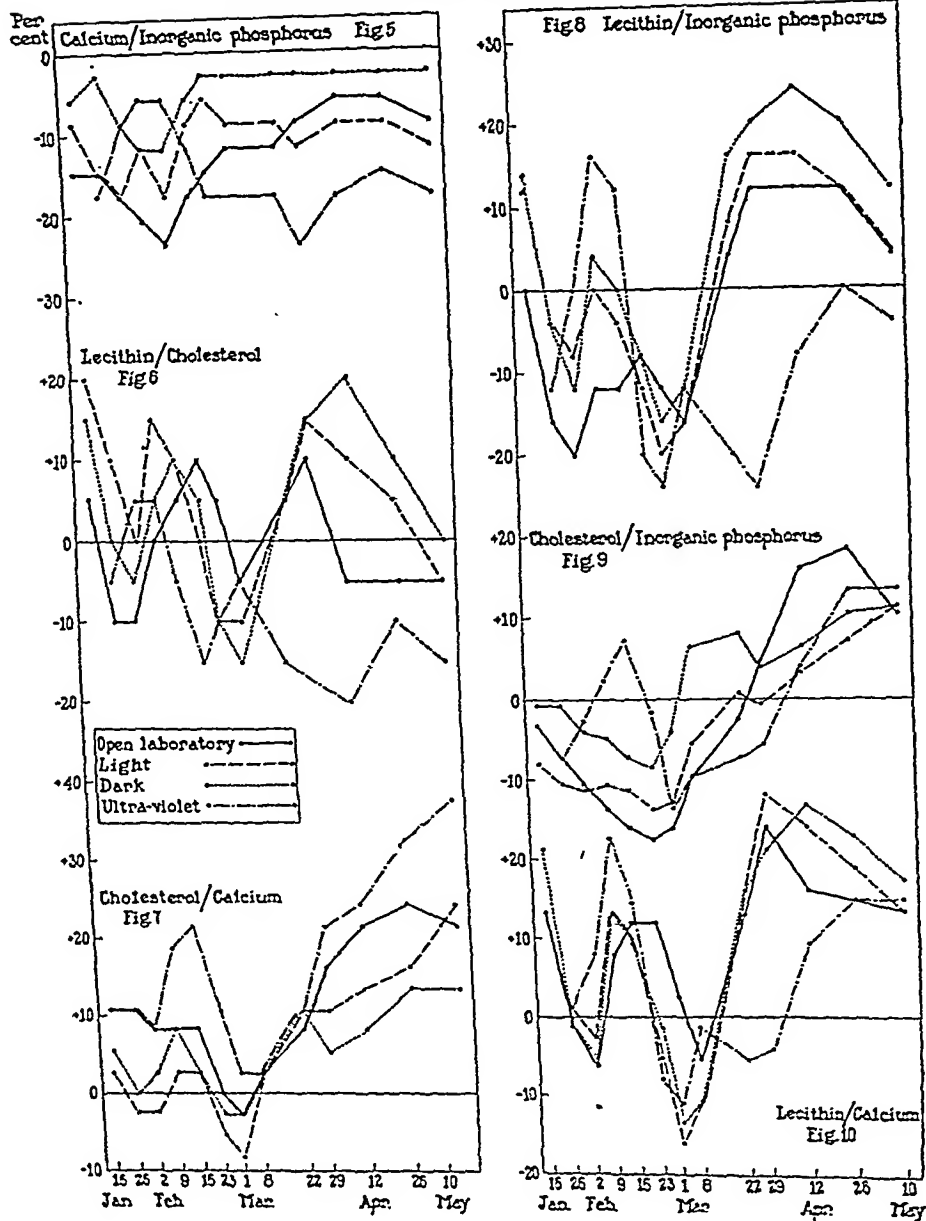
Group	Calcium	Phosphorus	Cholesterol	Lecithin	N
Open laboratory.....	15.3 ± 0.05	5.21 ± 0.01	62.7 ± 0.45	124.0 ± 1.33	150
Dark room.....	15.6 ± 0.05	4.83 ± 0.02	60.9 ± 0.38	126.0 ± 1.65	150
Light.....	15.5 ± 0.05	5.02 ± 0.02	59.8 ± 0.42	125.0 ± 1.35	150
Ultra-violet.....	15.3 ± 0.05	5.26 ± 0.04	65.9 ± 0.51	120.0 ± 1.22	140



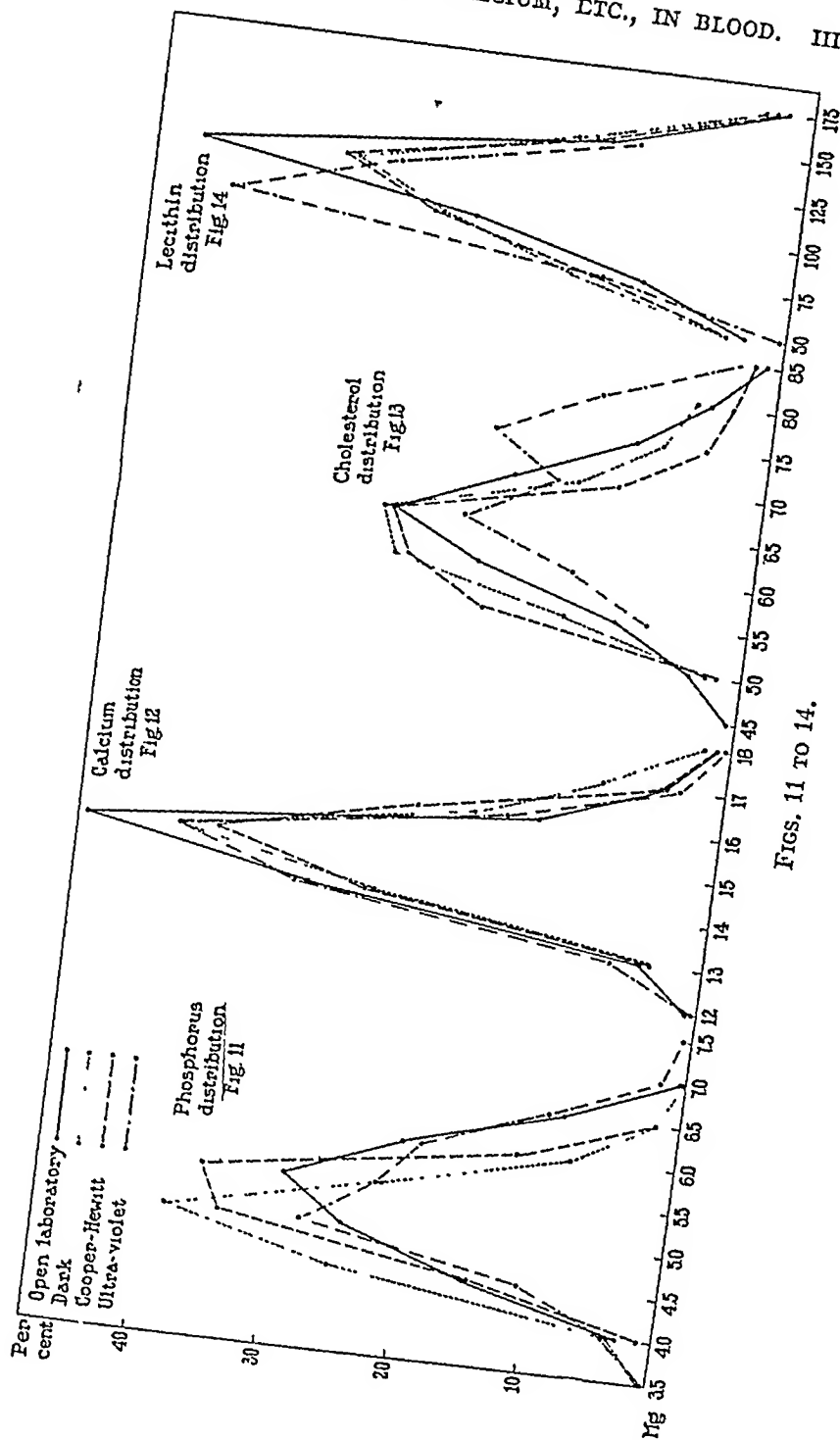
FIGS. 1 AND 2.



Ratio values
Per cent deviation from standard means (3)



FIGS. 5 TO 10.



FIGS. 11 TO 14.

DISCUSSION AND CONCLUSIONS.

Variation.

Calcium.—The element calcium (Text-fig. 1) proved to be the most stable and least variable of the 4 blood constituents. This was true for each of the 4 groups of animals.

The trend of calcium in the blood serum of animals living in the open laboratory showed a gradual decrease from the beginning of the experiment on Jan. 5 until about Feb. 9. Throughout the next 3 weeks the calcium in this group of animals increased in amount until on Feb. 29 it reached its highest level. From this high value the calcium decreased until about Mar. 20 at which time it had reached a more or less stable level which was maintained during the remaining 2 months of the experiment.

The animals living in the dark and those exposed to Cooper Hewitt light showed similar trends for the first 3 months of the experiment but exhibited a degree of variation as to the general levels of calcium metabolism. These groups of animals showed first an increase in calcium and then a rather rapid decline, reaching their minimum level at the same time as those animals living in the open laboratory, the dark group slightly lower, and the light slightly higher than those living in the open laboratory. From the low values of Feb. 9, the calcium of both groups of animals increased in amount, reaching their maximum on Feb. 29. Throughout the initial period of the experiment, the animals in the dark room maintained a lower level of calcium than those exposed to the Cooper Hewitt light. This difference, however, was reversed, and from Feb. 29 throughout the remaining time of the experiment, the animals living in the dark room maintained a higher level of calcium than any of the others, while the light group followed closely the trend of the animals living in the open laboratory.

The animals exposed to ultra-violet rays showed first, a slight increase of calcium and then a decrease, this decrease occurring at a time when the trend of calcium for the 3 other groups was on the increase. The ultra-violet group reached its highest level of calcium on Mar. 6, this occurring at a time when the trend of all other groups was downward. From this time until the end of the experiment, the calcium for the ultra-violet group continued to decrease.

During the last month of the experiment the 4 groups of animals exhibited 4 distinct levels for the calcium content of blood serum. The animals living in the dark room had the highest amount of calcium, those exposed to Cooper Hewitt light ranking next with a smaller amount, the third group, those animals living in the open laboratory with a slightly lower amount of calcium than the light group, while the least amount of calcium during this last 4 weeks' period was found to be in the animals exposed to the ultra-violet light.

Inorganic Phosphorus.—The inorganic phosphorus of the blood serum (Text-fig. 2) ranked next to calcium with a higher degree of variation. This fact held true for each of the 4 groups of animals. The greatest variation occurred in the group of animals living in the open laboratory while the animals in the dark room showed the least variation. The most abrupt changes in inorganic phosphorus were noted to occur in the blood serum of those animals that were exposed to ultra-violet light.

Analyzing the curves for the individual groups, it will be noted that with the animals living in the open laboratory there occurred a gradual increase in inorganic phosphorus which reached its maximum amount on Feb. 16. This gradual increase was not unlike that which occurred in the group of animals previously reported (2) and it will also be noted that this maximum occurred at a time when the previously reported group gave its minimum values, *i.e.*, at about 6 weeks from the beginning of the experiment. From this high value, the inorganic phosphorus in the serum of animals living in the open laboratory exhibited a gradual and uninterrupted decrease, reaching its lowest level on Apr. 12. The last 3 determinations gave only slight increases in inorganic phosphorus over this minimum value.

The animals exposed to Cooper Hewitt light showed the same initial rise in inorganic phosphorus as the animals living in the open laboratory. It will be noted, however, that at the beginning of the experiment the light group maintained a decidedly lower level than those in the open laboratory, but the maximum values for the 2 groups of animals occurred at about the same time. From this high point on Feb. 16, the inorganic phosphorus showed a gradual decrease which continued until Apr. 12, the last 3 determinations giving increasing amounts over this minimum value. At the beginning of the experi-

ment the level of inorganic phosphorus for the light group was far below that of the open laboratory group, but this order was reversed on about Mar. 20, and from this time until the end of the experiment the light animals maintained a higher level of inorganic phosphorus than those living in the open laboratory.

Except for a slight decrease at the beginning of the experiment, the trend of inorganic phosphorus in the serum of animals living in the dark room was the same as that found in the light and open laboratory groups. With the dark group the maximum value, which occurred Feb. 23, came 1 week later than the maximum of either the light or open laboratory groups. From this high amount, the inorganic phosphorus in the blood serum of the dark animals gradually decreased in amounts until about Apr. 9, when the minimum for the entire experiment was obtained. It will be noted that the general level of inorganic phosphorus maintained by the dark group was below that of the light, and except for one determination, that of May 19, was far below the level maintained by the animals living in the open laboratory. The animals living in the dark room showed the least variation.

The inorganic phosphorus of the ultra-violet group exhibited trends and variations in a direction opposite to that of the 3 other groups of animals. At the beginning of the experiment this group of animals gave the highest value but during the succeeding 4 weeks the inorganic phosphorus decreased, reaching its minimum on Feb. 21. It may be noted that this continued decrease occurred over a period of time when the 3 other groups of animals were exhibiting trends of increasing amounts. From this minimum, the ultra-violet group showed a gradual increase in inorganic phosphorus and except for the 2 determinations on Mar. 10 and 20, reached their maximum on Mar. 30. This high value for the ultra-violet group was reached at a time when the 3 other groups had more or less stabilized at their respective minimum levels. From this maximum, the inorganic phosphorus showed decreasing amounts for the 2 next determinations and was followed by increasing amounts for the 2 last examinations.

During the last 10 weeks of the experiment, the 4 groups of animals maintained distinct levels of inorganic phosphorus, these levels being independent and except for one value found in the dark group, showed no overlapping. The highest level of inorganic phosphorus was main-

tained during the last 10 week period by the ultra-violet group followed in order by the light group and animals living in the open laboratory, with the dark group at the lowest level.

Cholesterol.—The variability exhibited by the cholesterol (Text-fig. 3) in the blood of the 4 groups of animals was practically the same as that of inorganic phosphorus.

The group of animals living in the open laboratory showed a gradual decrease in cholesterol from the beginning of the experiment, reaching their minimum on Feb. 29. This low value occurred at a time when animals living out of doors, *i.e.*, just received from the dealer, were maintaining their maximum level of cholesterol for the entire year. From this minimum the cholesterol in the blood of the open laboratory group rapidly increased, the maximum value occurring on Apr. 19. The 2 last determinations showed only a slight decrease from this maximum value.

At the beginning of the experiment the group of animals exposed to the Cooper Hewitt light showed the least amount of cholesterol, but during the last 10 weeks gave values which were only slightly less than those for the group living in the open laboratory. The light group at the beginning of the experiment showed a gradual decrease in cholesterol which was followed by a slight increase during February, and a second decrease which reached its minimum on Feb. 29. Throughout this entire period the light group showed the least cholesterol of any of the 4 groups. During the remainder of the experiment the trend of cholesterol for this group of animals was upward, except for the one determination on Mar. 29, and a level below the open laboratory group was maintained until the last examination was made.

The animals living in the dark room maintained a level of cholesterol which in general was between the values shown by the light and open laboratory groups, but during the last 4 weeks was just below the animals exposed to Cooper Hewitt light. From the beginning of the experiment the cholesterol decreased until a minimum was reached on Feb. 23. This was followed by a gradual increase until Mar. 9 at which time a marked decrease in cholesterol was noted. The following determinations, however, gave values which represented the maximum for this group for the entire time of the experiment.

The group of animals exposed to the ultra-violet light gave 2 values

for cholesterol at the beginning of the experiment which were slightly below that obtained for the group in the open laboratory. The ultra-violet group then showed a rapid increase in cholesterol, reaching a maximum for this period on about Feb. 14. This increase occurred at a time when the cholesterol of all other groups was decreasing. The trend of cholesterol following this high point was downward until Mar. 10 when it reached its minimum. From this minimum the cholesterol exhibited a very rapid increase which continued throughout the remainder of the experiment. It will be noted that except for the 2 values at the beginning of the experiment and the one obtained on Mar. 20, the general level of cholesterol for the ultra-violet animals was markedly higher than that for any of the 3 other groups of animals. For the last 6 weeks of the experiment, in the order of decreasing levels, the ultra-violet group ranked first; the group living in the open laboratory came next with practically 10 per cent less cholesterol; the light and dark groups followed with the light group slightly higher in cholesterol than the dark.

Lecithin.—The abruptness and degree of variation proved to be greater for lecithin (Text-fig. 4) than for any of the 3 other blood constituents. This fact held true for each of the 4 groups of animals. The animals living in the open laboratory, the dark and light groups, listed in the order of increasing values for lecithin at the beginning of the experiment, showed a decreasing trend which reached a minimum on Feb. 2. From this point, the 3 groups exhibited a rather abrupt increase in lecithin, the group living in the open laboratory reaching its maximum about Mar. 1 which was 10 days later than the maximum for the light or dark groups. During the following 4 weeks, there occurred another decrease in lecithin in each of these 3 groups of animals which terminated with minimum values for the 3 groups on Mar. 10.

During the last 8 weeks of the experiment, the 3 groups of animals showed a rapid increase in lecithin, the dark group showing the greatest amount throughout this period; the light group next with slightly less; and the open laboratory group with the lowest level for the 3 groups.

The animals exposed to the ultra-violet light maintained the highest level of lecithin during the first part of the experiment, but during the

last 8 weeks they were the lowest of the 4 groups. Beginning with lecithin far below the 3 other groups, the ultra-violet animals showed a rapid increase which reached its maximum about Feb. 21. This occurred at a time when the 3 other groups were exhibiting decreasing trends. From this high point, the lecithin decreased and reached a minimum about the same time as the light and dark groups, this being the lowest point attained by the ultra-violet group throughout the experiment. A slight increase in lecithin was found at the next examination, but this was followed by 2 determinations of decreasing values. It will be noted that this decrease occurred at a time when the 3 other groups exhibited their greatest per cent increase. From this low level the lecithin in the ultra-violet group gradually increased and at the last determination was slightly higher than for the group living in the open laboratory or the group exposed to Cooper Hewitt light. The general level of lecithin maintained by the ultra-violet animals throughout the last 8 weeks of the experiment, however, was markedly lower than that of the 3 other groups.

Considering the calcium, inorganic phosphorus, cholesterol, and lecithin throughout the course of the experiment in all groups of animals, it will be noted that the curves representing the trend of these 4 blood constituents may in general be divided into two periods. The first 8 weeks of the experiment showed the greatest degree of irregularity in trend and frequency of variation. This period, as suggested by Brown (3) may be called the period of adjustment or accommodation to changed environment. In this experiment the period of accommodation was about 2 weeks longer than that required by the group of animals previously reported (2) but this difference in time may be attributed to the difference of environmental conditions. The second period, comprising the last 8 weeks of the experiment, was characterized by relative stability. During this last 8 weeks, each group of animals exhibited and maintained more or less distinct levels for each of the 4 blood constituents which can only be explained on the basis of differences in light environment, since all other factors for the 4 groups of animals were, as far as possible, the same.

The mean values for calcium, inorganic phosphorus, cholesterol, and lecithin maintained by these 4 groups of animals over the entire period of the experiment are presented in Table VI. These values

show differences of the same general order as those indicated in the text-figures.

Ratios.

The 6 possible combinations of calcium, inorganic phosphorus, cholesterol, and lecithin in the form of ratios are presented in Text-figs. 5 to 10 inclusive. In order that each group might serve as a control for the others all values were reduced to terms of a percentage deviation from a standard mean as explained above.

Each of the 6 ratio text-figures is divisible into two sections as in the case of the curves showing the variations in the amounts of the 4 substances. The first period of some 8 weeks represents the period of accommodation to the changed environment. It will be noted here, as in the previous curves, that all ratios during the first 8 weeks exhibited wide degrees of variation and rather abrupt changes, and that it was not until the last 8 weeks of the experiment that any degree of stability was maintained by all groups of animals. Since the true effect of the conditions studied is shown best by the results obtained during the last 8 weeks of the experiment, the discussion will be limited to this period.

The most consistent and the highest calcium-inorganic phosphorus ratio (Text-fig. 5) was maintained by the animals living in the dark room. While both calcium and inorganic phosphorus showed a certain degree of variation during this period of the experiment, the ratio between the 2 substances was practically constant. A change in relative position took place between the light and open laboratory animals during the last 5 weeks' period. At the beginning, the light group exhibited a higher ratio of calcium-inorganic phosphorus, but fell slightly below the open laboratory groups after Mar. 22. This change in ratio value was due primarily to the drop in inorganic phosphorus of the animals in the open laboratory below that maintained by the light group.

The lowest calcium-inorganic phosphorus ratio during this period of the experiment was maintained by the animals exposed to ultra-violet light. This low ratio was due to both a decrease in calcium and an increase in inorganic phosphorus. The order into which these 4 groups of animals aligned themselves with respect to the calcium-

inorganic phosphorus ratio was first, the animals living in the dark with the highest ratio; second, the open laboratory group; third, the light group; and fourth, the ultra-violet group with the smallest ratio value. It will be noted that all values were lower than those for the standard group (3). This was due mainly to a difference in calcium values.

Stability in the lecithin-cholesterol ratios (Text-fig. 6) for all groups of animals did not occur until some 10 days after the calcium-inorganic phosphorus ratios had been established at more or less definite levels. The delay in lecithin-cholesterol stabilization was due chiefly to the variation which occurred in the cholesterol content of the blood during this period.

The highest lecithin-cholesterol ratio was maintained by the animals living in the dark room. At the beginning of the last 8 weeks' period, the ratio maintained by the light group coincided with that of the dark, but during the final 5 weeks, there was a separation of the 2 groups, the ratio of the light falling below that of the dark group.

The animals living in the open laboratory showed the least variation in the lecithin-cholesterol ratio. With the beginning of the last 8 weeks' period, this group gave ratio values below both the dark and light groups. The final ratios were somewhat lower than that maintained by either the light or dark groups.

The animals exposed to the ultra-violet light maintained the lowest lecithin-cholesterol ratio. This low value was maintained throughout the last 8 weeks. Listing the 4 groups of animals in the order of the relative magnitude of the levels maintained, the animals in the dark room came just with the highest lecithin-cholesterol ratio; the light group second; the open laboratory group came third; and the ultra-violet animals fourth with the smallest ratio values. The low position of the ultra-violet group was the result of the high cholesterol (Text-fig. 3) and the comparatively low lecithin values (Text-fig. 4) exhibited throughout this last 8 weeks' period.

The cholesterol-calcium ratios (Text-fig. 7) for the 4 groups of animals showed levels which were just the reverse of the lecithin-cholesterol ratios. The ultra-violet groups maintained the highest cholesterol-calcium ratio throughout the last 8 weeks; the open laboratory group, except for the last value, came next with a slightly lower

ratio; then the light group; and the animals in the dark with the lowest value for this period of the experiment.

The ratio of lecithin to inorganic phosphorus (Text-fig. 8) was highest in animals living in the dark room. The light and open laboratory groups came next with a slight decrease in ratio values, and it will be noted that at the time of the 2 last determinations these groups gave values which coincided.

The ratio of lecithin to inorganic phosphorus maintained by the ultra-violet group was markedly below that found in any other group of animals. This low ratio was due mainly to a high inorganic phosphorus, but the lecithin was also lower than in the other animals.

The cholesterol-inorganic phosphorus ratio (Text-fig. 9) showed marked delay in stabilization with more overlapping of the trends than any of the other ratios. This condition may be ascribed to the wide variation in cholesterol content of the whole blood than to the inorganic phosphorus which exhibited a much greater degree of stability and maintained a definite separation of levels for each of the 4 groups of animals.

The animals living in the open laboratory gave the highest cholesterol-inorganic phosphorus ratio except for the last determination at which time these animals were the lowest of the 4 groups. The ultra-violet group, which at the beginning of this period gave values below those for all other animals, came next with a slightly lower ratio; the final value, however, was slightly above that obtained for the 3 other groups.

The animals in the dark room exhibited a greater stability with respect to their cholesterol-inorganic phosphorus ratio than the other groups of animals. While at the beginning of this period the ratio values of the dark group were well above those of other animals, the subsequent increase was less than that of the open laboratory and ultra-violet groups, both of which rose to a higher level than the dark group. At the last determination, the ratio for the animals in the dark coincided with that found for the light group. The animals exposed to Cooper Hewitt light maintained a cholesterol-inorganic phosphorus ratio below that of the dark group throughout the experiment, except as pointed out, at the time of the last examination when these 2 ratios were the same.

The results of the 6 ratio curves (Text-figs. 5 to 10) with respect to relative position of each group of animals during the last 8 weeks of the experiment are presented on a comparative basis in the following table, the figure 1 representing the highest and 4 the lowest ratio value.

Group	Ca./P.	Lec./Chol.	Chol./Ca.	Lec./P.	Chol./P.	Lec./Ca.
Dark room.....	1	1	4	1	2	1
Cooper Hewitt.....	3	2	2	2	3	2
Open laboratory.....	2	3	3	3	1	3
Ultra-violet.....	4	4	1	4	4	4

These results show definitely that the group of animals in the dark room and those exposed to ultra-violet light occupied diametrical positions with respect to their ratio levels. Except for 2 ratios, the animals in the open laboratory maintained values next to that of the ultra-violet group, while the light group gave results nearer those of animals in the dark.

Distribution.

The distributions of all values obtained for each of the 4 substances studied are presented according to groups in Text-figs. 11 to 14 inclusive. These curves are similar in form to those previously reported (1) except for the one curve representing the distribution of cholesterol for the ultra-violet group of animals. The irregularity in this curve is probably due to the extreme high values obtained for these animals during the last 6 weeks of the experiment. The differences in position of the other curves correspond to the results presented in Text-figs. 1 to 4.

Correlation.

The coefficients of correlation for the 6 possible combinations of calcium, inorganic phosphorus, cholesterol, and lecithin are presented in Table VI. Section (a) gives the results obtained by calculating the correlation coefficients using mean values for each group and, therefore, presents the relationship with respect to trend. Except for

$r_{P.Ca.}$ which gives a positive correlation for the 4 groups of animals, the remaining 5 correlations were the same with respect to sign as those presented in a previous report (1). The highest degree of correlated trend was found in the $r_{Chol. Lec.}$ of animals exposed to the Cooper Hewitt light. In the $r_{P. Lec.}$ the ultra-violet group gave the highest degree of correlated trend.

In section (b) of the same table the correlation coefficients are calculated on the basis of the mean values maintained by each individual animal throughout the experiment. The signs of the $r_{P.Ca.}$ in this section are negative, while in the previous section they are positive. This is due to the fact that while individual animals maintained negative relationships in their corresponding sigma values, the general trend of the mean values for calcium and inorganic phosphorus tend to parallel each other in degree and direction of variation.

The correlation coefficients in section (c), calculated on the basis of a comparison of single determinations with the means for the entire series, give values which in the majority of cases are the same as section (a).

Throughout the 3 sections it will be noted that the $r_{Chol.Lec.}$ were all positive in sign, the magnitude of the coefficients varying somewhat with respect to the different groups and method of calculation. These results correspond with those reported in a previous paper (1) and seem to indicate that the 2 lipid substances, cholesterol and lecithin, parallel each other in variation and trend.

The relationship existing between the inorganic phosphorus of the blood serum and the lecithin of the whole blood is of especial interest. Reviewing the results so far obtained with respect to these two substances, it will be noted that lecithin in all cases has proved to be the most variable constituent and has shown the most abrupt and most frequent change of trend. The coefficient of variation and standard deviation of inorganic phosphorus emphasize the stability of this substance when compared to the lipid fraction. It seems probable, therefore, that there is a reciprocal relationship existing between inorganic phosphorus and lecithin and also the lipid fraction may serve as a reservoir or source of supply for the inorganic form.

The results show that animals when brought into the laboratory pass through a period of adjustment or accommodation which in this

experiment covered a period of about 8 weeks. The mechanism by which these effects are induced is unknown.

The results obtained from this experiment are interpreted as furnishing evidence that exposure to the ultra-violet light for a comparatively brief period daily results in a definite change in levels of calcium, inorganic phosphorus of the blood serum and cholesterol and lecithin of the whole blood. Animals excluded from the effects of all light, as far as is practicable, maintained levels of the 4 blood constituents diametrical to the group of animals exposed to ultra-violet rays. The animals living in the open laboratory and those exposed to the Cooper Hewitt light exhibit levels of the 4 blood constituents which are somewhat similar in position, but both groups maintained values just between those of the dark and ultra-violet groups of animals.

SUMMARY.

Experiments are reported in which it was shown that the calcium, inorganic phosphorus, cholesterol, and lecithin in the blood of normal rabbits were influenced by 4 types of light environment. The results of the experiments seem to warrant the following conclusions:

1. Animals exposed to the ultra-violet light for a brief period each day give results which are diametrical to those obtained for animals living in total darkness.

2. The results obtained for animals exposed to the Cooper Hewitt light and for those living in the open laboratory are somewhat similar but occupy a position between those of the dark and ultra-violet groups.

3. Animals do not develop immediately the characteristic effects of a particular environmental condition, but pass through a period of accommodation which varies somewhat with different environmental conditions.

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REACTIONS OF RABBITS TO NON-HEMOLYTIC STREPTOCOCCI.

II. SKIN REACTIONS IN INTRAVENOUSLY IMMUNIZED ANIMALS.

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In previous papers it has been shown (1, 2) that rabbits inoculated in practically any manner, except intravenously, with sufficiently large doses of certain non-hemolytic streptococci developed a condition of tissue hypersensitiveness. This was made evident by the occurrence of secondary reactions at the site of the primary inoculation, by the presence of corneal sensitivity after a certain period, by much larger reactions following intracutaneous re-inoculation than occur in normal animals inoculated with similar doses of culture; and finally by death of many of the animals following intravenous inoculation with amounts of culture well tolerated by normal rabbits. It was also shown that if the primary inoculation of the animals had been by the intravenous route, using amounts of culture and time intervals comparable with those employed in the hypersensitized (*i.e.*, intracutaneously inoculated) rabbits, these intravenously inoculated animals responded with none of these reactions of hypersensitiveness. From such observations one might have concluded that they reacted to re-inoculation in the same manner as normal animals; but on closer study it was found that their reactivity differed from that of either normal or hypersensitive animals. The object of this communication is to present the macroscopic evidence that these intravenously inoculated rabbits react differently than do normals to intracutaneous inoculation.

EXPERIMENTAL.

The methods employed were comparatively simple: animals were inoculated intravenously with varying amounts of centrifugate of 18 to 24 hour blood broth cultures of non-hemolytic streptococci, and

after varying periods were inoculated into the previously depilated skin with decreasing amounts of similar cultures of homologous microorganisms. The test doses were usually 10^{-1} cc., 10^{-2} cc., 10^{-3} cc., and 10^{-4} cc., the first in 0.1 cc. volume, the other three in 0.05 cc. volumes, as it was found that with these amounts distinct differences in reaction of the various animals were made quite clear. At the time of the skin testing a group of normal control animals was inoculated in a similar manner; hence the strength of the culture used in any given experiment was determined.

Experiment 1.—Two rabbits, Q515 and Q516, were inoculated intravenously on Feb. 5 and Feb. 15, 1926, with the centrifugate of 5 cc. of culture of *Streptococcus* V92/0/11. On Feb. 19 they, together with 3 normal controls, Q531, Q532, Q533,

TABLE I.

Average Size of Lesions following Intracutaneous Inoculation of Rabbits Previously Inoculated Intravenously, Compared with Controls.

	No.	Size of inoculum		
		10^{-1} cc.	10^{-2} cc.	10^{-3} cc.
		mm.*	mm.	mm.
Intravenously inoculated.....	2	33	15	Negative
Normals.....	3	39	20	9

* Indicates average sum of 2 longest diameters of the lesions.

were inoculated intracutaneously with 10^{-1} cc., 10^{-2} cc., and 10^{-3} cc. of the homologous strain. The lesions were measured and described daily. 2 days later a striking difference in the reactions of the 2 groups of animals was evident: At the site of the 10^{-1} cc. inoculation of Rabbits Q515 and Q516 there were red, hard, shotty lesions; at the site of the 10^{-2} cc. lesions there were flat, barely palpable, pink, maculopapules, and where 10^{-3} cc. had been injected there was no macroscopic evidence of injury. The controls at this time showed larger and softer lesions resulting from inoculation of 10^{-1} cc. and 10^{-2} cc. doses and with one exception also distinct soft papules at the site of the 10^{-3} cc. inoculation.

Because all of the rabbits received intravenous inoculations 7 days later the subsequent development of the lesions was probably altered, hence the main differentiation between the 2 groups appears in the physical characteristics of the reactions and in the average size of comparable lesions in the 2 sets of animals, as shown in Table I.

The results of a different time interval between the immunizing and testing doses are shown in Experiment 2.

Experiment 2.—Each of 4 animals, Q380, Q381, Q385, and Q386, received intravenously the centrifugate of 10 cc. of blood broth culture, V92/0/10. 20 days later the reactivity of their skins, together with that of 4 controls, was tested with doses of 10^{-1} cc., 10^{-2} cc., and 10^{-3} cc. of homologous culture. The results are shown in Table II.

TABLE II.

Comparison of Skin Reactivity of Rabbits Intravenously Immunized 20 Days Previously with That of Normal Animals.

	Rabbit No.	Sum of diameter of lesions			Secondary reaction	Retested after 10 days; sum of diameter of lesions		
		Size of inoculum				Size of inoculum		
		10 ⁻¹ cc.	10 ⁻² cc.	10 ⁻³ cc.		10 ⁻¹ cc.	10 ⁻² cc.	10 ⁻³ cc.
		mm.	mm.	mm.		mm.	mm.	mm.
Immunized intra-venously	Q380	55	22	16	0	44	23	16
	Q381	30	30	16	0	37	26	22
	Q385	46	26	24	0	52	24	16
	Q386	32	23	16	0	33	29	17
Average.....		41	25	18		41	25	18
Normal	Q415	36	22	16	+	76	30	24
	Q416	37	20	0	+	46	27	18
	Q417	49	25	27	0	47	25	16
	Q418	50	19	0	0	46	27	21
Average.....		43	21	11		54	27	20

It is at once obvious that the differences in the size of primary reactions between the intravenously inoculated group and normals was not so marked as in Experiment 1. Neither was the nodular character of the lesions of the immunized group so marked as in the first experiment. Nevertheless, 2 of the 4 normal rabbits showed secondary reactions at the sites of the 10^{-1} cc. and 10^{-2} cc. inoculations, while none of the immunized animals showed secondary reactions.

It thus appears that differences in the interval between immunizing inoculation and skin testing had a distinct influence on the character of the reaction following intracutaneous inoculation. When this period was short, as in Experiment 1, the differences between the

immune and normal animals were greater than in Experiment 2, where an interval of 20 days had elapsed. It is probable that the relatively slight immunity that followed the single intravenous injection of 10 cc. of streptococci was passing off by this time. Still it was sufficient to prevent the development of secondary reactions, and an accompanying general hyperergy; for the immunized group retested 10 days later showed practically the same sized lesions as when first tested, while the controls similarly retested showed distinctly larger lesions.

The effect of more prolonged continuous intravenous inoculation is brought out better in Experiment 3.

Experiment 3.—A group of 7 animals, R651, R652, R653, R654, R655, R657, and R658, were selected for immunization with culture of *Streptococcus* V110A. Five of them were inoculated intravenously as follows: 1st day 1 cc., 3rd day 1 cc., 5th day 2 cc., 7th day 4 cc., 12th day 5 cc. Two received only 1 cc. each on the 11th and 14th days respectively. On the 16th day all were tested with intracutaneous inoculations of 10^{-1} cc. and 10^{-2} cc. of blood broth culture of homologous streptococci; 7 normal rabbits were tested with similar doses. On the 18th day the first 5 rabbits of the first group each received the centrifugate of 4 cc. of culture intravenously and the other 2 cc. On the 27th day all received 4 cc.; thus the immunization was continued during the period in which secondary reactions might have been expected to appear.

The results of these intracutaneous inoculations are shown graphically in Charts 1 and 2. In Chart 1 are given the curves formed from daily measurements of the sum of the 2 longest diameters of the lesions, while in Chart 2 the volumes of the lesions resulting from the 10^{-2} cc. inoculations are indicated.¹ A distinct difference is immediately evident. Only 3 out of the 7 rabbits inoculated intravenously showed secondary reactions, but 2 of these had received only 2 small inoculations before the skin testing; the third showed only a slight and late secondary reaction. Six out of the 7 normal animals, on the other hand, developed secondary reactions, which in most instances usually appeared about the 8th to the 10th day; none of the immune animals, on the other hand, showed secondary reactions before the 14th day. The primary reactions of the 2 groups of animals also showed similar differences in consistency and size to those previously noted. These differences in size are brought out more strikingly by comparing their

¹ The method of calculating these volumes is given in a previous paper (2).

volumes as shown in Chart 2, where not only the greater initial size of the primary lesions, but also that of the secondary reactions in the

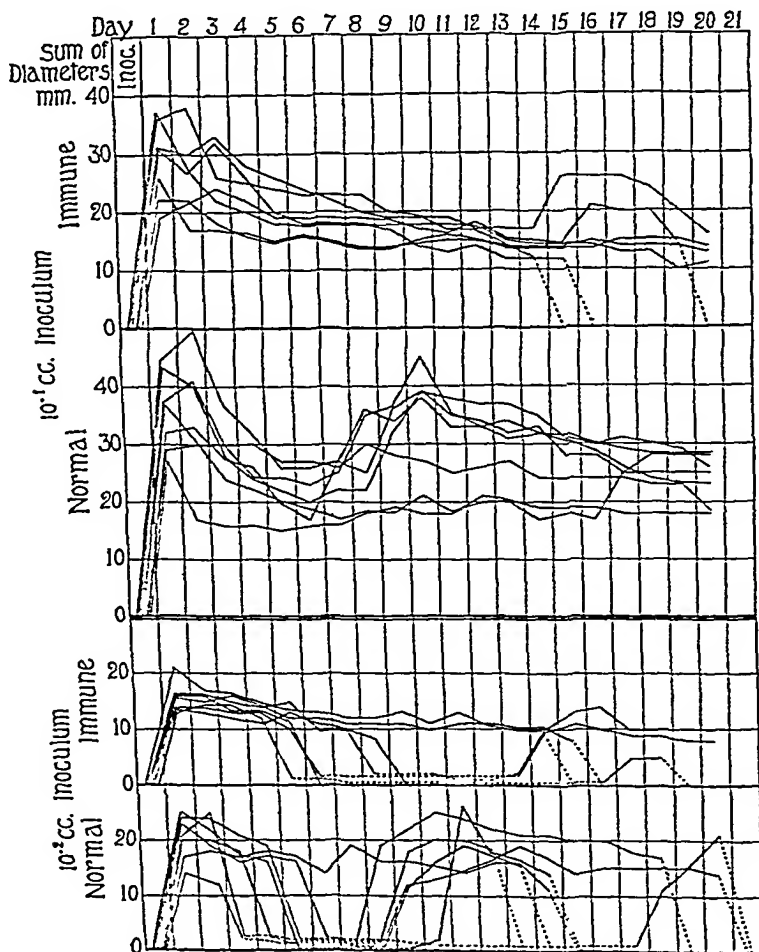


CHART 1. Comparison of sizes of lesions of immune and normal rabbits following intracutaneous inoculation with *Streptococcus* V110A.

normal animals is made evident. The immunized animals showed only relatively small increases in their lesions at the time of their secondary reactions, while the normal animals showed very marked in-

creases. This experiment indicates how necessary it is not only to measure the diameters, but also to pay careful attention to the thickness of these lesions. The nodular character of the primary reactions in the immune group compared with softer lesions in the normal animals was observed here as in previous experiments.

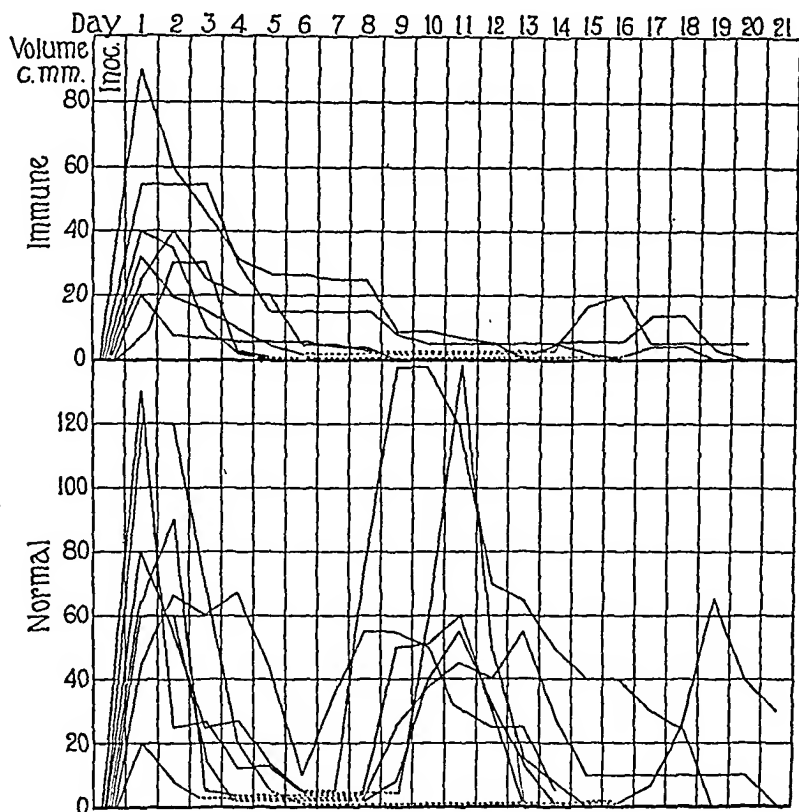


CHART 2. Comparison of volumes of lesions of immune and normal rabbits following intracutaneous inoculation with 10^{-2} cc. of blood broth culture of *Streptococcus* V110A.

In view of the fact that such differences in the tissue response could be demonstrated when animals were previously inoculated intravenously with living organisms, it was decided to investigate whether or not a similar alteration in responsive capacity could be detected if a bacterial fraction were used as the immunizing agent. It had been previously demonstrated by Lancefield (3) that the nucleoprotein ex-

tract of green streptococci was the only fraction with a definite antigenic capacity. She, therefore, prepared a fairly large quantity of nucleoprotein of *Streptococcus* V92 with which a group of rabbits was immunized intravenously, as indicated in Experiment 4.

Experiment 4.—Eight rabbits were injected intravenously with a normal saline solution of green streptococcus nucleoprotein as follows: 1st, 2nd and 3rd days, 10 mg. each day; 9th, 10th, 11th and 12th days, 20 mg. each day; 17th, 18th, 19th and 20th days, 30 mg. each day. On the 26th day each rabbit received intracutaneous inoculations with the centrifugate of blood broth culture of *Streptococcus* V92 in the following amounts, 5 cc., 5 cc., 10^{-1} cc. and 10^{-2} cc.; 4 normal controls were similarly inoculated. The curves showing the reactions of 4 of the immunized animals compared with the 4 controls are shown in Charts 3 and 4. Only the curves of the 10^{-1} cc. and 10^{-2} cc. lesions are given because the intensity of the response to 5 cc. of culture was so marked in all rabbits that differences in the 2 groups were not made so evident as with the smaller inocula.

While differences in the diameters of the primary lesions in the 2 groups of animals were not very marked, it became apparent, as the evolution of the lesions was followed, that there was a distinct difference in the response of the animals. All of the normal controls showed well marked secondary reactions in all of their lesions, while only 3 of the 8 immunized animals developed secondary reactions in the smaller lesions; and in 2 instances these were slight and delayed. The striking difference in the character of these secondary reactions is brought out in Chart 4, where the volume of the 10^{-2} cc. lesions of 4 of the immunized rabbits is compared with similar lesions in the 4 controls.

While the average volume of the primary lesions was 69 c.mm. in the immunes compared with 94 c.mm. in the controls, that of the secondary reactions was 25 c.mm. in those developing these reactions compared with 230 c.mm. in the controls. It is evident, therefore, that immunization with nucleoprotein over a period of approximately 4 weeks had altered the rabbits' type of response towards intracutaneous inoculation in the direction of a diminished intensity of reaction. It must be realized that the amount of nucleoprotein used represented large bacterial growths, and that probably a similar period of immunization with a corresponding amount of vaccine would have been more efficacious. The main point to be gathered from this ex-

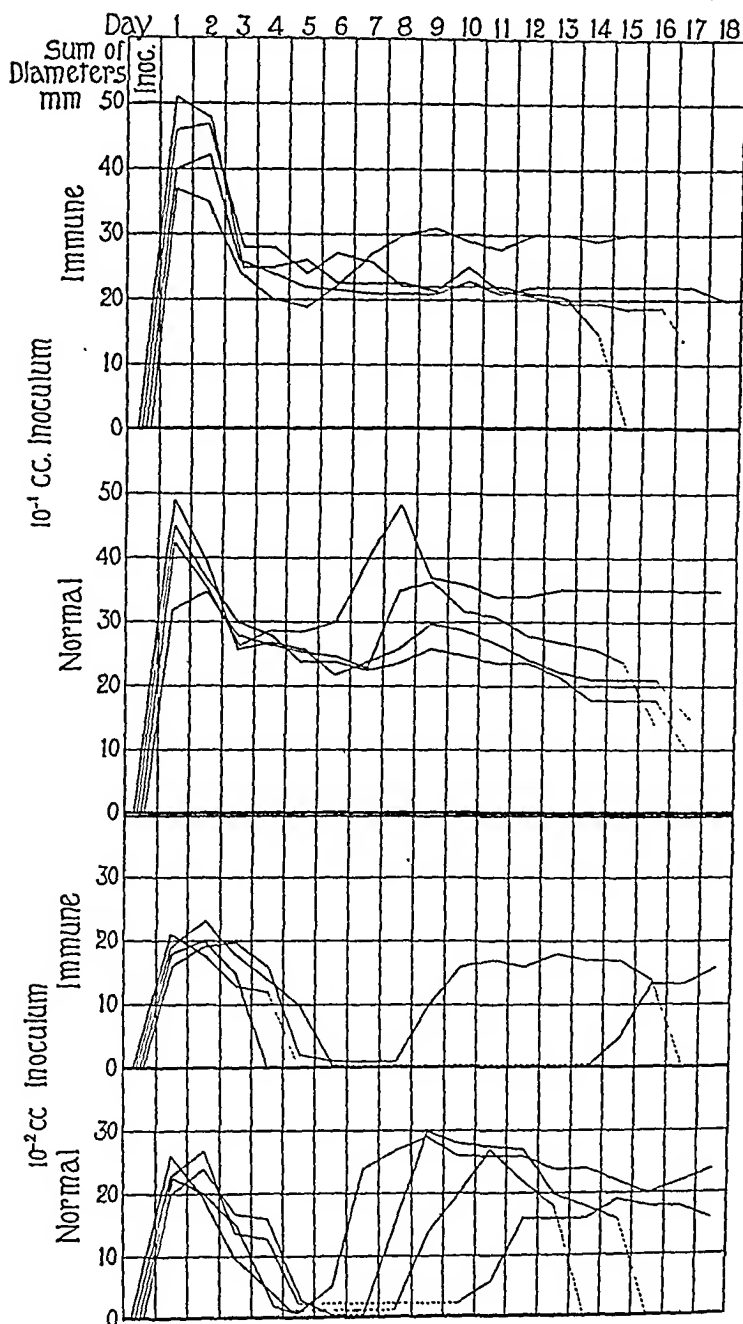


CHART 3. Comparison of sizes of lesions of rabbits immunized with nucleoprotein of green streptococci and normal rabbits following intracutaneous inoculation with *Streptococcus* V92.

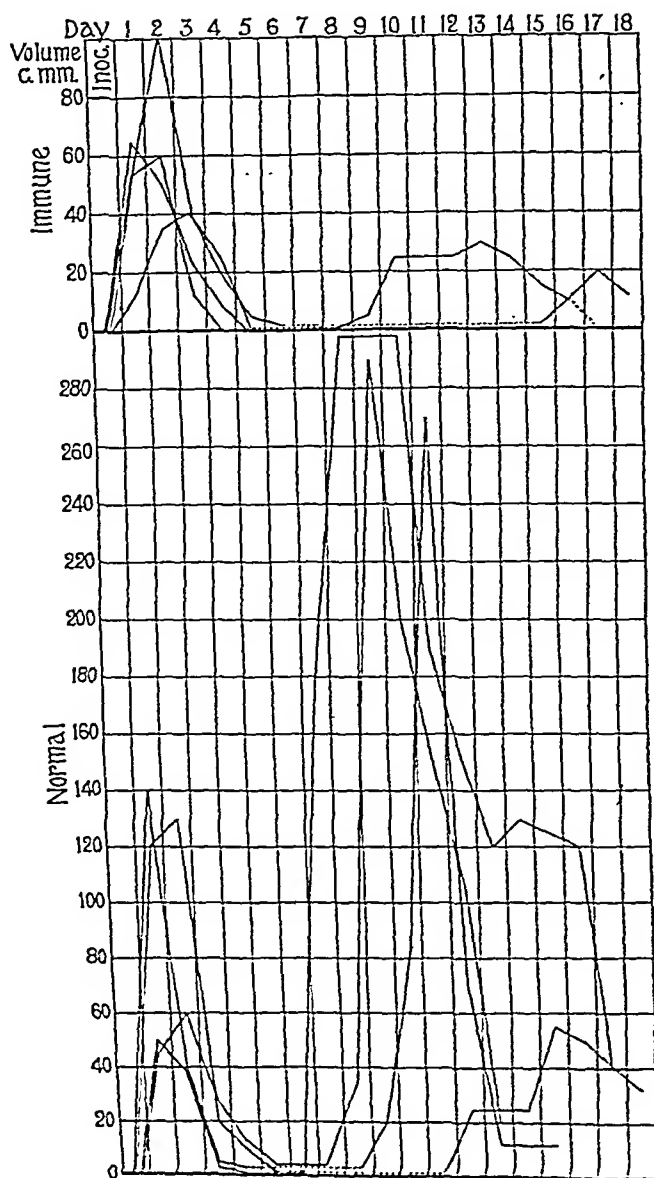


CHART 4. Comparison of volumes of lesions of rabbits immunized with nucleoprotein of green streptococci and normal rabbits following intracutaneous inoculation with 10^{-2} cc. of blood broth culture of *Streptococcus* V92.

periment was that the nucleoprotein fraction carried the immunizing substance.

As a final experiment the effect of prolonged intravenous immunization was studied as shown in Experiment 5.

Experiment 5.—Twelve rabbits were inoculated intravenously over a period of 9 weeks. In the beginning 0.5 cc. of whole blood broth culture of *Streptococcus* V110A was employed. This was gradually increased to 2 cc. after 10 days; but the rabbits began to lose weight so rapidly that immunization was discontinued for a week, then recommenced with 1 cc. of heat killed culture. This, in turn, was gradually increased until 5 cc. doses were given; subsequently immunization with living culture was resumed without depressing the rabbits. During the succeeding period of 4 weeks 9 inoculations were given by gradually increasing the dose from 1 cc. to 4 cc. of living blood broth culture. At the end of this period the sera of all the rabbits showed a precipitin titer of 1:4000 to 1:8000 when tested against nucleoprotein prepared from *Streptococcus* V110A. The agglutinin titer of the sera could not be determined because of spontaneous agglutination of the cultures.

After this course of 9 weeks' immunization the skin reactivity of all the rabbits together with that of 4 normal controls was tested with *Streptococcus* V110A/0/5 in 6 doses of from 10^{-1} cc. to 10^{-6} cc. None of the animals showed reactions at the sites of the 10^{-4} cc., 10^{-5} cc., and 10^{-6} cc. inoculations. The maximum and average sizes of the lesions in the 3 larger lesions in all of the animals are shown in Table III; also the volumes of the 10^{-2} cc. and 10^{-3} cc. lesions are given.

While quantitative variations in the response of the different animals occurred, there was almost a uniform tendency for the immunized animals to show smaller lesions than the controls. The ratio of the averages in the immune group compared with the normals at the sites of the different inocula was 10^{-1} cc. 2:3; 10^{-2} cc. 1:2; 10^{-3} cc. 2:3. When volumes of lesions are compared, just as in previous experiments, the differences are even more striking. In the 10^{-2} cc. lesions the average ratio was 1:3, and in the 10^{-3} cc. lesions 4:9. The daily notes of the consistency of the lesions also brought out striking differences. The immune animals had very hard shotty lesions, particularly at the sites of 10^{-2} cc. inoculations, while the normal animals showed some edema with only moderately infiltrated lesions from the 1st to the 3rd days. These differences were so striking that the immune animals could be readily differentiated from the controls by simple comparison of the size and character of the corresponding lesions.

None of the immune animals showed secondary reactions in any of

their lesions, while 3 out of the 4 controls developed secondary reactions. It may be objected that the differences in the size of the 2 groups tend to invalidate the comparisons, but all of 4 other normal rabbits inoculated with a corresponding culture 2 days later developed primary lesions and secondary reactions similar to these 4 controls; so it may

TABLE III.

Comparison of Skin Reactivity of Rabbits Immunized Intravenously Over a Long Period with Normal Controls.

	Rabbit No.	Sum of diameters of lesions			Volume of lesions	
		Size of inoculum			Size of inoculum	
		10 ⁻¹ cc.	10 ⁻² cc.	10 ⁻³ cc.	10 ⁻¹ cc.	10 ⁻² cc.
		mm.	mm.	mm.	c.m.m.	c.m.m.
Immunized intra- venously	1119	40	17	12	98	15
	1120	35	17	v. and p.*	90	v. and p.*
	1122	42	21	13	117	17
	1123	34	19	12	108	6
	1124	24	16	9	50	2
	1125	40	25	15	165	22
	1131	38	16	12	10	14
	1135	47	18	10	54	2
	1136	53	28	20	188	60
	1138	38	20	14	106	20
	1139	33	20	15	100	12
	1140	42	28	15	157	22
Average.....		39	20	12	110	16
Normal	1253	70	39	20	517	55
	1254	52	27	15	150	10
	1255	53	34	18	315	48
	1256	78	38	17	423	45
Average		63	37	17	334	37

* Non-measurable, visible and palpable.

be safely concluded that these controls represented the average response of normal animals at this period. This experiment, therefore, indicates that prolonged intravenous immunization causes a group of animals to react to subsequent intracutaneous inoculation in a more uniform manner than does short immunization such as was carried out with living streptococci in the earlier experiments, or with streptococcus nucleoprotein as in Experiment 4.

DISCUSSION.

It is common knowledge that previous inoculation alters the reaction of an animal towards an infectious agent; in fact upon this knowledge are based our efforts towards the prevention and cure of infectious diseases where the various techniques of immunology are employed. In certain conditions the outstanding defensive agents are easily demonstrable in the body fluids in the form of antitoxins, opsonins or similar antibodies. In other diseases, where the invasive microorganisms have relatively low virulence, or elaborate comparatively little exotoxin, the defensive mechanism seems to reside in the cells rather than in the humors of the body. But in most infections both humoral and cellular factors apparently play a rôle; and in proportion as one or the other predominates, so, in part at least, may differences in the focal manifestations of infection and resistance be explained.

Following Pirquet's (4) observations and introduction of the term allergy, studies of the local manifestations of reinfection have assumed a constantly increasing importance, until at the present day an enormous literature has grown up about this word. It seems to be quite generally accepted that the phenomenon of allergy represents a very intense local effort on the part of the animal to limit the activity of infectious agents or foreign substances to the site where they gain entrance into the animal's body. It is also obvious that local reactions are quite different following the introduction of such agents as vaccinia, tubercle bacilli, egg albumin or primin into suitably allergized animals or men. Re-inoculation with vaccine virus results in smaller local injury than occurs at the time of first inoculation, while reinjection with egg albumin leads to more marked local reaction, which increases in intensity with increase of circulating antibodies (5). Re-inoculation with tubercle bacilli, on the other hand, is followed by destructive localized tissue reaction, the Koch phenomenon, without any corresponding increase of antibodies in the blood serum. Repeated treatment of the skin with primin, a nitrogen free ether extract of primrose results in a type of allergy made manifest by an eczematous inflammation of the skin (6). In these four examples of allergy the differences in local response may be explained in part by differences in the nature of the antigen or inciting agent. Following the introduction of tubercle bacilli into the body tubercles are formed

with an accompanying general state of tuberculin allergy regardless of the presence of demonstrable circulating antibodies. Egg albumin, on the other hand, may be introduced intravenously into an animal without producing gross focal injury; nevertheless with the development of antibodies a state of allergy ensues. It is therefore apparent, as pointed out by Zinsser (7), Doerr (8), Coca (9), and others, that the allergy induced by bacteria and by coagulable proteins is different. In practically all instances in which a tuberculin-like allergy is induced this follows some focal tissue reaction resulting from injury by the respective micro-organism.

But, in so far as we are aware, no comparison has previously been made of the type of tissue reaction which follows the introduction of bacteria into the body so that in the one instance gross lesions are produced and in the other no macroscopic lesions are formed. One difficulty attending such experimentation has been due to the fact that most bacteria, previously used in the study of allergy, induced focal lesions regardless of the route employed for inoculation. But with non-hemolytic streptococci, such as we have employed, these conditions could be more easily controlled. Strains with which it was possible to induce a condition of tuberculin-like allergy when they were injected into the tissues, could also be used to decrease the animal's tissue reactivity when they were injected intravenously in suitable doses. Ordinarily streptococci of this type quickly disappear from the blood stream without producing gross lesions; but in some instances, especially when large doses are employed, the animals develop endocarditis or arthritis. In one rabbit of Experiment 3, in which an arthritis of the wrist had followed the early intravenous inoculation, the skin test inoculation was followed by lesions of the hypersensitive rather than of the immune type. This isolated example taken in conjunction with other experiments previously reported (2) supports the viewpoint that focal lesions are probably necessary for the development of hypersensitiveness of infection.

Another point worthy of note is that intravenously immunized rabbits when inoculated intracutaneously with suitable doses show lesions of a different character than do either normal or hypersensitive animals, lesions that have little if any edema and are hard and firm after 24 to 48 hours. In fact these lesions show much less change in size

after 2 days than do the lesions in other types of animals. The larger inocula often are followed in 4 or 5 days by globular sac-like areas persisting for weeks, and smaller inocula produce small hard persistent nodules. In other words, the entire and complete reaction seems to take place much more quickly in the immune than in other states of allergy. The microscopic comparison of the different types of lesions will be reported in a later communication.

Finally, for purposes of completeness it should be noted that certain rabbits give reactions following intracutaneous inoculation which differ from any of those previously described by us. These reactions are usually less marked than those of normal animals, are soft, have very little color, fade rapidly, and do not show secondary reactions. They occur in rabbits which appear sick, either due to an overwhelming infection from streptococci, or from any other cause. These animals are usually emaciated and have a skin which is wrinkled, gray and lacking in tone. We have designated these reactions "cachectic," and after a little experience have learned to recognize them within 2 or 3 days following intracutaneous inoculation. Obviously the inclusion of such animals in any group is to be avoided for they vitiate statistical comparisons.

Within recent years it has become evident to many observers that different states of resistance towards a given bacterium could be indicated by differences in cutaneous reactivity. For example, a patient showing well marked positive reaction to intracutaneous injections of tuberculin may lose this skin reactivity during an attack of measles or other infectious disease, or if he becomes cachectic from a neoplasm or even from an overwhelming tuberculous infection. This has been designated as negative anergy, and corresponds with what we call cachectic reaction. It has also been observed that an animal may show decreasing skin reactivity to a certain fixed dose of a testing bacterial extract while it is becoming more resistant to a general infection with the same bacterium (10). This state has been designated as positive anergy, and probably corresponds with our designation, immune reaction. It seems probable, then, that the term allergy will have to be qualified in some manner, if it is to be continued as a means of expressing the idea of increased resistance accompanied by over-reaction of the tissues. For this reason we have at times used the term *hyperergy* which has been employed by a number of German

pathologists (11, 12) to indicate a hyper-reactivity of the tissues, and the terms immune or cachectic to indicate gross decreases in reactivity according to the manner in which this decreased tissue reactivity is induced.

SUMMARY.

Rabbits immunized intravenously with living culture or nucleoproteins of non-hemolytic streptococci react to subsequent intracutaneous inoculations with homologous streptococci with smaller and harder lesions than are shown by normal animals similarly inoculated; and they do not develop the general manifestations of hypersensitiveness such as are shown by animals previously inoculated into the tissues with the same cultures.

A rabbit may react to intracutaneous inoculation with non-hemolytic streptococci in one of four ways, depending on whether it is normal, hypersensitive, immune or cachectic. Most normal animals show a secondary reaction about 10 days after inoculation with suitable strains of non-hemolytic streptococci; hypersensitive, allergic, or hyperergic animals show much larger lesions than do normals with the corresponding doses of the same streptococci, and practically never show secondary reactions; immune animals show smaller and harder early lesions and usually do not have secondary reactions if they are fairly well immunized. Cachectic animals show very soft and rapidly fading primary reactions and no secondary reactions.

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TREATMENT OF EXPERIMENTAL ACUTE GENERAL PERITONITIS IN THE DOG WITH ILEOSTOMY AND SODIUM CHLORIDE SOLUTION.

By THOMAS G. ORR, M.D., AND RUSSELL L. HADEN, M.D.

(From the University of Kansas School of Medicine, Kansas City, Kansas.)

(Received for publication, November 21, 1928.)

Enterostomy as an aid in the treatment of peritonitis has attracted the attention of many clinicians in recent years. Since reports of its value have been somewhat conflicting we have made a study of the treatment of peritonitis by ileostomy from the experimental standpoint. In a recent publication we have reported the changes which occur in the blood chemistry in experimental general peritonitis (1). The blood chemistry has also been studied in this series of experiments. In addition to the treatment of general peritonitis with ileostomy a small series of dogs has been treated with both ileostomy and 1 per cent salt solution.

Normal dogs, weighing from 7 to 15 kilos, were used in these experiments. Operations were done under complete ether anesthesia with aseptic technic. General peritonitis was produced by ligating the appendix with tape. At the same operation the ileum was divided 6 inches above the cecum, the distal portion invaginated with a purse string suture and the proximal portion brought to the surface through the wound and sutured to the fascia and skin. In some cases a tube was placed in the lumen of the ileum to insure drainage. Blood was drawn daily and sometimes twice daily from the jugular vein for chemical analysis. The animals were kept in a warm room in metabolism cages and given water *ad libitum*.

The non-protein nitrogen was determined by the method of Folin and Wu (2), the urea nitrogen by the Van Slyke and Cullen modification of the Marshall method (3) and the carbon dioxide-combining power by the method of Van Slyke (4). The chlorides were determined on the tungstic acid filtrate as suggested by Gettler (5).

OBSERVATIONS.

In order to produce a general peritonitis, the appendix was freed at its base and snugly ligated with tape. Any animal developing

TABLE I.

Appendix Ligated to Produce Peritonitis. Ileum Sectioned 6 Inches above Cecum and Proximal Open End Sutured to Skin for Drainage.

Dog No.	Day after operation	Blood			CO ₂ -combining power	
		Amount per 100 cc.				
		Total non-protein nitrogen	Urea nitrogen	Chlorides		
		mg.	mg.	mg.	vol. per cent	
1	0	34.1	18.2	510	36.2	Operation
	1	48.2	25.2	460	40.0	
	2	91.5	47.6	390	38.1	Autopsy—general peritonitis
2	0	27.7	12.1	440	36.2	Operation
	1	31.7	13.5	420	36.2	
	2	94.0	52.3	350	—	Autopsy—general peritonitis
3	0	33.0	17.2	460	34.3	Operation
	1	40.0	17.7	450	34.3	
	2	136.8	67.7	330	—	Autopsy—general peritonitis
4	0	41.6	22.4	470	30.5	Operation
	1	77.0	39.2	380	34.3	
	2	165.0	65.3	370	—	Autopsy—general peritonitis
5	0	24.8	14.4	430	36.2	Operation
	1	20.2	9.8	400	34.3	
	2	41.0	19.6	380	38.1	
	3	50.8	17.7	400	36.2	
	4	107.0	66.3	370	28.7	
	5 (a.m.)	208.0	91.0	320	29.6	
	5 (p.m.)	190.0	83.5	330	20.2	Autopsy—general peritonitis
6	0	26.8	12.1	460	31.5	Operation
	1 (a.m.)	59.5	37.3	270	—	
	1 (p.m.)	104.0	51.3	320	29.0	
	2 (a.m.)	131.0	74.7	290	30.5	
	2 (p.m.)	114.0	80.7	300	37.2	
	3	159.0	80.7	280	40.9	Autopsy—general peritonitis
7	0	28.5	14.5	460	34.3	Operation
	1	42.8	17.8	410	41.9	
	2	115.0	59.8	400	32.4	
	3 (a.m.)	140.0	77.1	330	36.2	
	3 (p.m.)	254.0	129.8	290	—	Autopsy—general peritonitis

TABLE I—*Concluded.*

Dog No.	Day after operation	Blood			CO ₂ -combining power	
		Amount per 100 cc.				
		Total non-protein nitrogen	Urea nitrogen	Chlorides		
		mg.	mg.	mg.	vol. per cent	
8	0	57.8	31.7	500	37.2	Operation
	1	39.5	20.5	470	38.1	
	2	70.0	36.8	390	36.2	
	3	61.0	28.0	350	43.8	
	4	63.0	29.8	340	44.7	
	5	88.3	35.9	350	34.3	
	6	148.0	94.8	260	—	Autopsy—general peritonitis
9	0	28.0	8.8	530	43.8	Operation
	1	23.3	11.2	500	30.5	
	2 (a.m.)	59.0	32.2	410	22.1	
	2 (p.m.)	107.0	56.0	410	29.6	
	3	103.0	63.0	350	22.1	Autopsy—general peritonitis

complications was discarded. Autopsy was done in every case to determine the extent of peritoneal involvement. More than 50 per cent of the dogs developed a localized abscess at the site of the appendix or got well. Only those having a definite general peritonitis are recorded here.

In the first series, which included nine animals (Table I), the length of life varied from 2 to 6 days with an average of 3 1/9 days. In a previous study of eight animals with general peritonitis without drainage of the ileum (1) the average length of life was 4 1/8 days.

The changes in the chemistry of the blood were in all cases similar to those previously found in general peritonitis, showing an increase in the non-protein and urea nitrogen, a decrease in the chlorides and no constant change in the carbon dioxide-combining power.

The second series of animals with ligated appendix and ileostomy (Table II), was treated early with 40 cc. of 1 per cent sodium chloride solution per kilo of body weight. These dogs lived from 4 to 18 days or an average of 10 1/3 days. With the administration of salt solution, the changes in the chemistry of the blood were not marked. In some

TABLE II.

Appendix Ligated to Produce Peritonitis. Ileum Sectioned 6 Inches above Cecum and Proximal Open End Sutured to Skin. Treatment with 1 Per Cent Sodium Chloride Solution.

Dog No.	Day after operation	Blood			CO ₂ -combining power	
		Amount per 100 cc.				
		Total non-protein nitrogen	Urea nitrogen	Chlorides		
		mg.	mg.	mg.	vol. per cent	
1	0	29.1	9.3	410	31.5	Operation, 400 cc. 1% NaCl
	1	31.6	9.8	480	36.2	" " " "
	2	—	—	—	—	" " " "
	3	38.0	14.9	540	21.9	" " " "
	4	21.1	9.3	530	29.6	" " " "
						Autopsy—general peritonitis
2	0	54.6	35.0	530	31.5	Operation, 408 cc. 1% NaCl
	1	38.9	14.0	490	30.5	" " " "
	2	22.5	7.0	470	30.5	" " " "
	3	22.9	7.9	470	29.6	" " " "
	4	27.0	9.3	460	34.3	" " " "
	5	20.0	7.5	430	32.4	" " " "
	6	27.0	14.0	480	31.5	" " " "
						Autopsy—general peritonitis
3	0	23.3	9.8	410	24.0	Operation, 400 cc. 1% NaCl
	1	24.6	14.4	420	29.6	" " " "
	2	24.0	11.6	460	32.4	" " " "
	3	21.4	14.0	490	32.4	" " " "
	4	22.4	14.9	500	22.1	" " " "
	5	22.8	8.8	540	25.6	" " " "
	7	20.7	8.8	550	25.6	" " " "
	8	20.4	9.3	580	27.5	" " " "
	9	21.1	7.0	580	28.5	" " " "
	10	22.5	9.3	630	21.1	" " " "
	11	20.7	10.7	610	16.4	" " " "
	12	22.9	8.4	600	18.3	" " " "
	14	23.0	7.9	620	22.1	" " " "
	16	30.0	9.8	650	14.5	" " " "
	18	41.6	36.4	890		" " " "
						Autopsy—general peritonitis

TABLE II—*Concluded.*

Dog No.	Day after operation	Blood			CO ₂ -combining power	
		Amount per 100 cc.				
		Total non-protein nitrogen	Urea nitrogen	Chlorides		
		mg.	mg.	mg.	vol. per cent	
4	0	24.2	17.2	440	32.4	Operation, 380 cc. 1% NaCl
	1	25.2	14.9	460	14.5	" " " "
	3	21.6	12.1	480	29.6	" " " "
	4	20.8	11.2	510	32.4	" " " "
	5	20.7	13.5	430	27.5	" " " "
	6	19.0	9.8	570	25.6	" " " "
	7	20.3	8.9	430	21.1	" " " "
	8	41.0	18.9	460	22.1	" " " "
						Autopsy—general peritonitis
5	0	51.8	26.1	450	31.5	Operation, 352 cc. 1% NaCl
	1	28.9	18.2	480	36.2	" " " "
	2	20.3	17.2	480	36.2	" " " "
	3	20.7	14.0	470	37.2	" " " "
	4	21.4	11.6	430	38.1	" " " "
	5	22.2	12.1	490	32.4	" " " "
	6	20.7	10.2	480	17.4	" " " "
	7	20.5	11.2	510	25.8	" " " "
	8	22.9	10.2	580	15.5	" " " "
	10	23.0	11.2	550	16.4	" " " "
	12	30.9	14.9	550	17.4	" " " "
	13	28.5	15.8	530	—	" " " "
	14	79.8	41.1	420	—	" " " "
6	0	30.9	11.6	480	32.4	Operation, 280 cc. 1% NaCl
	1	34.9	10.2	410	29.6	" " " "
	2	30.9	11.2	420	35.3	" " " "
	3	—	—	—	—	" " " "
	4	31.9	13.0	470	—	" " " "
	5	27.7	12.6	490	36.2	" " " "
	6	25.7	14.0	540	27.5	" " " "
	7	23.4	11.6	510	32.4	" " " "
	8	28.2	16.3	560	31.5	" " " "
	9	23.8	15.4	500	32.4	" " " "
	10	—	—	—	—	" " " "
	11	53.6	32.2	470	—	" " " "
12	52.0	78.4	490	11.1	" " " "	
						Autopsy—general peritonitis

cases there was a terminal rise in the non-protein and urea nitrogen and in one case, living 18 days, a definite rise in the chlorides. The carbon dioxide-combining power showed no constant change.

DISCUSSION.

In this study of general peritonitis, treated with ileostomy from the beginning, no beneficial results are noted. Life is not prolonged by this method of treatment nor are the typical changes in the blood chemistry noted in experimental general peritonitis prevented.

The animals treated with 1 per cent solution of sodium chloride hypodermically in addition to ileostomy lived three times as long as those having no salt solution. The administration of the sodium chloride solution was quite effective in preventing the chemical changes of the blood noted in animals without such treatment. The results of treatment with sodium chloride in this study are similar to those previously recorded in the treatment of high intestinal obstruction (6).

CONCLUSION.

1. In experimentally produced general peritonitis drainage of the gut by ileostomy 6 inches above the cecum has no beneficial effect.

2. Animals with experimentally produced general peritonitis treated with ileostomy plus 1 per cent sodium chloride solution live three times as long as those not given the salt solution.

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THE RAPID SHALLOW BREATHING RESULTING FROM PULMONARY CONGESTION AND EDEMA.

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(From the Surgical Laboratories, Massachusetts General Hospital, Boston.)

(Received for publication, December 12, 1928.)

Experimental studies by Dunn (1) demonstrated that multiple embolism of the pulmonary arterioles and capillaries is followed by a temporary inhibition of respiration succeeded by rapid shallow breathing. Dunn attributed this phenomenon to the stimulation of sensory nerve endings in the lungs. This explanation was favored by the fact that the injection of starch produced no alteration in the respiratory rate when both vagi had been previously sectioned.

Attracted to this problem by its bearing upon the occurrence of rapid and shallow respiration in lobar pneumonia, Binger and Moore and their associates (2-6) have carried out a carefully planned series of experiments. Their studies chiefly concern the effects of the embolism of pulmonary arterioles and capillaries produced by the intravenous injection of starch grains under varying conditions. These observations were supplemented by other experimentally induced modifications of the pulmonary circulation. In their conclusion (6) to this instructive series of papers they state: "Since rapid and shallow breathing is not the result of (1) anoxemia, (2) increased $p\text{CO}_2$ and hydrogen ion concentration of the serum, (3) restriction of pulmonary vascular bed by nearly half, (4) increase in resistance to the flow of blood to and from the lungs, (5) the presence of starch grains in the lungs acting as a local irritant, it must be the result of the secondary pathological changes which occur in the pulmonary parenchyma following embolism. The nature of these changes, congestion and edema has been discussed elsewhere. Whether they operate directly on nerve endings or through their influence on lung volume and tissue elasticity is not certain." In their discussion the authors are inclined toward the hypothesis of direct irritation of the vagal nerve endings,

but wisely decline to theorize in the absence of more direct experimental evidence bearing on this point.

During the course of an investigation having quite another bearing, we have made certain observations which appear to demonstrate the occurrence of the usual signs of vagal stimulation in association with the experimental production of pulmonary congestion and edema. As a method completely different from those of Dunn or Binger was being employed, it seems desirable to briefly recount the results of our experiments.

Method.

A Drinker heart preparation (7) was made in a cat anesthetized with intravenous sodium-barbital (5 per cent solution). This procedure when complete leaves the heart exposed in a normally breathing animal, and is easily made by suturing the edges of the pericardium to the margins of an oval window created in the anterior wall of the chest by the removal of the sternum. After the closure of the chest and the resumption of normal respiration, ligatures were placed about the right branch of the pulmonary artery, and the group of right pulmonary veins respectively. The artery is readily reached from within the pericardium in that portion of its course which lies between the ascending aorta and the superior vena cava. The placing of a ligature about the right pulmonary veins is a more delicate procedure, as the thinness of their walls makes the liability to accidental puncture very real.

After both ligatures were in place they were tied so as to completely occlude the vessels, the one around the artery being tied first in order to avoid the effects of venous obstruction. An especially designed glass cannula was then inserted into the right branch of the pulmonary artery, distal to the point of ligation, and secured in place with a second ligature about the vessel. The cannula was connected by transparent rubber tubing to a glass reservoir which could be raised and lowered to effect changes in pressure. A T-tube leading from a point near the cannula was connected with a mercury manometer to record the pressure. The reservoir and tubing up to the cannula were filled with freshly drawn, heparinized blood from another animal, kept at body temperature.

Respiratory movements were recorded by a thread attached to the costal margin, moving a writing lever on the kymograph record. Systemic blood pressure was recorded by a citrated cannula in the right carotid artery. The blood pressure tracing served also to record pulse rate.

In such a preparation, the right lung is isolated from the rest of the circulation of the animal except for the amount of blood that may enter it through the bronchial artery. Ventilation of this lung is maintained by the normal respiratory movements of the animal as the lung is in its normal position in the closed pleural cavity. By changing the height of the reservoir connected to the cannula in the

right branch of the pulmonary artery, it is possible to alter the intravascular pressure in this isolated lung. Such changes may be produced without directly modifying the dynamics of the systemic circulation, or altering the gaseous exchange in the circulating blood. The latter is maintained by the normally functioning left lung and has been shown in other studies (8) to be adequate for the immediate needs of the animal.

EXPERIMENTS.

The following experiment illustrates the characteristic changes which have been observed in the respiratory rate and depth and in the systemic blood pressure and pulse rate following a sudden elevation of the intravascular pressure of the isolated right lung. As explained above the complete exclusion of this lung from the circulation of the animal assures that these effects are not due to pressure changes in the heart, variation of blood flow or alteration in the respiratory exchange.

Protocol. Experiment 1. Feb. 24, 1928. Circulatory Isolation of the Right Lung with the Production of Intravascular Pressure Changes.—2:30. Cat (2.7 kilos). Anesthesia induced by chloroform-ether to permit the injection of 19 cc. of sodium-barbital (5 per cent solution) into the saphenous vein. 3:08. Artificial respiration temporarily employed during the period when the chest is open. 3:45. Chest closed and residual air withdrawn from the pleural cavities. Artificial ventilation discontinued. 3:48. Blood pressure cannula inserted into the right carotid artery. 4:05. Ligatures placed about the right branch of the pulmonary artery and the right set of pulmonary veins. 4:20. Ligatures tied. Cannula inserted into the right branch of the pulmonary artery, and connected with a pressure reservoir filled with heparinized cat blood. 4:26. (Kymograph record, Fig. 1.) Reservoir elevated. 4:27. Reservoir lowered. 4:28. Reservoir elevated. 4:29. Reservoir lowered. 4:31. (Kymograph record, Fig. 2.) Reservoir elevated. 4:32. Reservoir lowered. 4:33. Experiment concluded. The heart showed no dilatation or change in appearance during the course of the experiment.

Autopsy.—No air in pleural cavities. The posterior and dependent parts of the lower lobe of the right lung were edematous. The rest of the lung was congested. A very small area of atelectasis was present in the right upper lobe. The left lung was of normal appearance and downy texture.

Sections of the kymograph tracing of this experiment are shown in Figs. 1 and 2. The results of the first elevation of the reservoir connected with the right pulmonary artery are recorded in Fig. 1. The rise in pressure is recorded in the change of level of line *D*, which indicates an elevation from 0 to 42 mm. of mercury. Following a latent period of 6 seconds, the systemic blood pressure (1) slowly

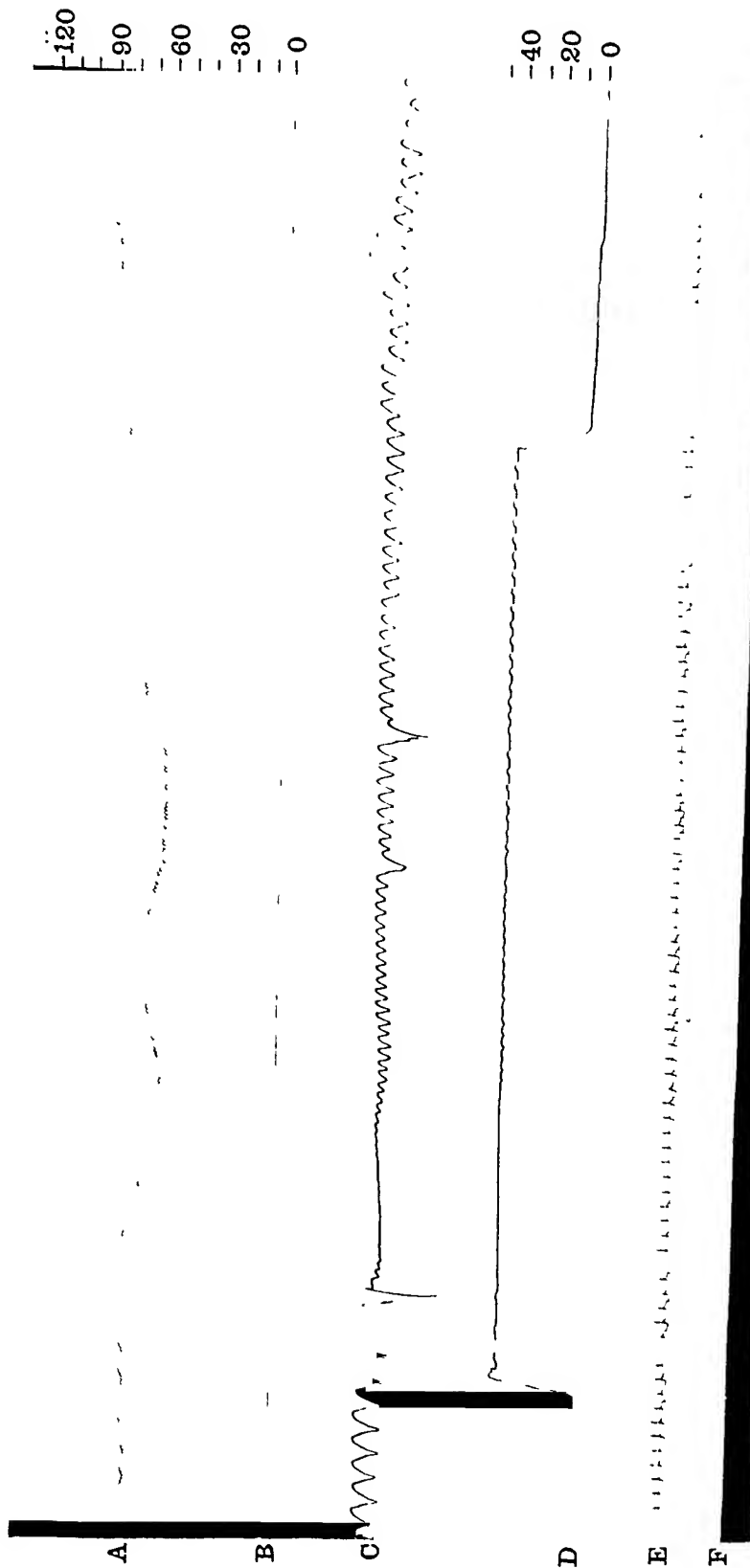


FIG. 1. Section of kymograph tracing from Experiment 1. *A*, systemic blood pressure in mm. of mercury recorded from right carotid artery. *B*, blood pressure base line. *C*, respiratory excursion of costal margin; down stroke of lever on inspiration. *D*, pressure in right branch of the pulmonary artery measured in mm. of mercury. *E*, time in 1 second intervals. *F*, signal marker indicating points when reservoir attached to the cannula in the right branch of the pulmonary artery was raised and lowered. The elevation of intravascular pressure in the isolated lung is followed by an apneic pause of respiratory excursions, succeeded by rapid shallow breathing. There is a simultaneous fall in blood pressure with slowing of the heart rate.

drops from 78 mm. of mercury to 60 mm. The pulse rate diminishes from 240 per minute to 204 per minute. The tracing of the respiratory excursions shows marked changes; a period of apnea lasting 8 seconds is followed by rapid and shallow respirations, the rate increasing from 47.5 to 75 per minute. These effects tended to disappear even before the reduction of pressure, although at the

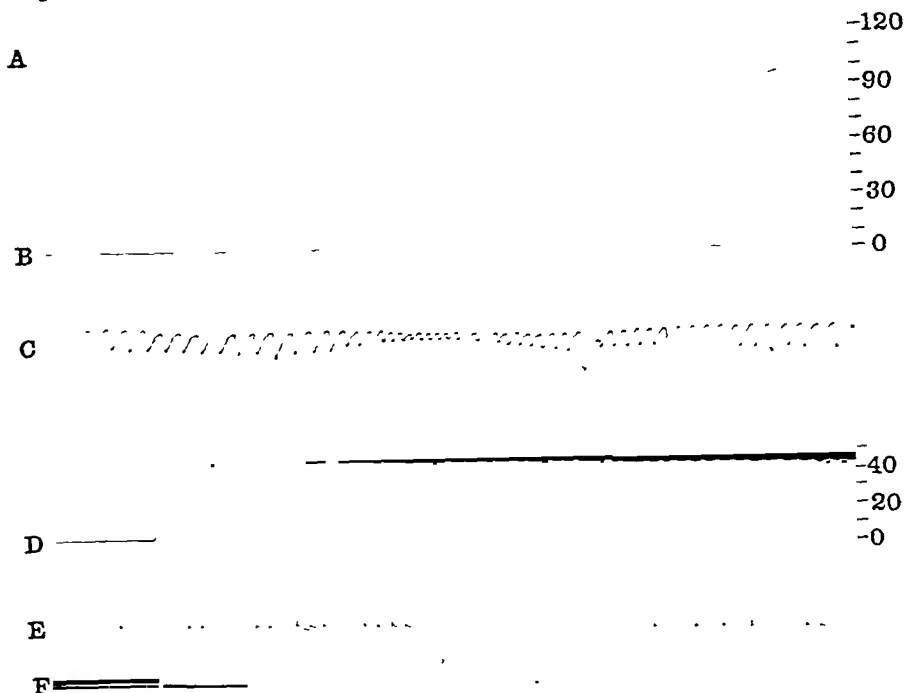


FIG. 2. Section of kymograph tracing from Experiment 1. Tracings as in Fig. 1. The elevation of pressure in the right branch of the pulmonary artery is followed by the institution of rapid shallow breathing without a period of apnea. The fall in blood pressure and slowing of heart rate is as in Fig. 1.

start of the second elevation, the respiratory rate was still increased over its previous value.

In the second experimentally produced rise in pressure, the tracing of which is not shown, similar changes took place. The pressure in the right pulmonary artery was raised from 0 to 38 mm. of mercury. Following a latent period of 7 seconds, the heart rate decreased from 240 to 228 per minute. The systemic blood pressure fell from 82 mm. of mercury to 77. The respiratory rhythm showed

no apneic pause, but the rate was increased from 45 to 80 per minute and the excursions became very shallow.

The third period is shown in Fig. 2. A rise in pressure of 42 mm. of mercury in the right pulmonary artery was followed by a latent period of 13 seconds. The systemic blood pressure dropped from 110 to 90 mm. of mercury, with a coincident slowing of cardiac rate from 252 to 228 contractions per minute. The respiratory rate increased from 45 to 90 per minute and became very shallow. As before, there was a tendency for the respiration to return to its original rate and depth before the pressure was reduced.

This experiment has been repeated fifteen times on six different cats with perfectly consistent results, *viz.*, a slowing of cardiac rate, a fall in systemic blood pressure and the institution of rapid shallow breathing, at times preceded by an apneic pause. It is unusual to be able to repeat the procedure in an individual animal with the same degree of success as was done in the experiment just described. Even here, the return to initial conditions between the changes in pressure was not complete. This is not surprising, as the congestive changes in the lung are presumably of a nature to produce a prolonged effect and not to permit of recovery during the period of the experiment.

DISCUSSION AND SUMMARY.

These experiments record the effects of the experimental production of pulmonary congestion and edema in a lung completely isolated from the general circulation, but with an intact nerve supply. The resulting changes are: a slowing of the heart rate, a fall in systemic blood pressure and a temporary inhibition of respiration succeeded by rapid shallow breathing. The pulse rate and blood pressure show a rapid and spontaneous return to initial conditions. The respirations show a partial but not a complete return to their former rate and depth. The effects on respiration are similar to those described by Dunn and Binger and Moore which follow multiple embolism of the pulmonary circuit with starch granules. The alterations in the pulse rate and blood pressure are characteristic of the effects of vagal stimulation. A chemical effect on the respiratory center is excluded by the nature of the preparation.

These results, therefore, add further evidence to support the hypothesis that the rapid shallow breathing attending congestion and edema of the lungs is due to the stimulation of nerve endings in the lungs.

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NON-HEMOLYTIC STREPTOCOCCI AND ACUTE RHEUMATIC FEVER.

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The association of specific non-hemolytic streptococci, of both the alpha and the gamma types, with acute rheumatic fever has been emphasized by several investigators in the past few years. The alpha type has been championed by Clawson (1), who was able to recover the organisms from the blood stream in a relatively high percentage of cases. Streptococci of the gamma type have been described by Small (2) and by Birkhaug (3), chiefly in relation to their presence in the throats of patients with acute rheumatic fever; with both investigators, however, their attention was originally directed to the organisms by the fact that they were recovered from the blood.

Granted that the etiologic factor of acute rheumatic fever were definitely known, it seems reasonable to assume that at least some advances might be made in the treatment of a disease in which, so far, therapeusis has been chiefly symptomatic and empirical. If streptococci of the alpha type are recovered fairly consistently from the blood stream in cases of rheumatic fever, or, if streptococci of the gamma type are occasionally found in the blood and are usually present in the throat, provided, in the latter case, that they do not occur in normal throats, either fact would furnish a lead worthy of extensive investigation.

Early in the fall of 1927 it was decided to take cultures from all available cases of acute rheumatic fever, and this has been done on 25 cases. The methods used have been such as to combine those of Clawson, Small and Birkhaug. Hitchcock (4) has recently published a paper which covers essentially the same ground in regard to the presence of non-hemolytic streptococci of the gamma type in the throats of rheumatic and non-rheumatic individuals.

*Methods.**Cases.*

The cases of acute rheumatic fever which were studied were carefully selected. Cultures were taken only from cases showing fever with a temperature above 100°F., manifestations of acute involvement of one or more joints and, as a rule, at least a moderate degree of leucocytosis. In those cases which had not received previous medical attention, efforts were made to obtain cultures prior to the administration of salicylates. The majority of the patients were in the wards of the Boston City Hospital, but cultures were obtained from 5 and 2 patients, respectively, through the kind cooperation of the Medical Services of the Massachusetts General and Peter Bent Brigham Hospitals.

Primary Cultures.

Blood.—The blood was withdrawn from an arm vein and the culture media inoculated at the bedside. As a rule, 50 to 60 cc. were withdrawn, though in several instances only 25 or 30 cc. were obtained.

One or 2 flasks containing 100 cc. of 0.2 per cent dextrose beef infusion broth (pH 7.5-7.7) were inoculated with 5.0 cc. of blood and 3.0 cc. were added to 50 cc. of beef infusion agar (pH 7.5-7.7), previously melted and cooled to $\pm 42^{\circ}\text{C}$. From the latter, after thorough mixing, 3 plates were poured. The remainder of the blood (10 to 40 cc.) was added to 1 or 2 sterile test-tubes and allowed to clot. The supernatant serum was pipetted off and saved for subsequent tests, and each clot was added to a flask containing 200 cc. of 0.2 per cent dextrose beef infusion broth (pH 7.5-7.7).

The blood agar plates were examined daily and discarded, in the absence of growth, at the end of 5 days. The flasks containing the blood and blood clot were examined daily during the 1st week for clouding of the medium or hemolysis. Smears were made and stained by the Gram method at weekly intervals. At the end of a month subcultures were made on blood agar plates, and, in the absence of growth, the flasks were discarded.

Throat.—Two swabs were used. Each was rubbed vigorously over all visible portions of the throat posterior to the anterior pillars of the tonsils. Each swab was rubbed, while twisting, over an area approximately 1.0 by 4.0 cm. on the surface of a blood agar plate (5 per cent rabbit's blood in beef infusion agar—pH 7.5-7.7). Proper dilutions were made on the remainder of the plate by smearing from this area with a sterile platinum loop.

Urine and Stool.—In several of the cases first studied cultures were made from fresh samples of urines and stools. The former were plated, undiluted, on blood agar plates. With the latter, a small bit was shaken up in a tube containing 0.2 per cent dextrose beef infusion broth, a loopful was spread on the surface of a blood agar plate and 2 more plates were made from the first by smearing with a sterile platinum loop. Suspicious colonies were transferred to dextrose beef infusion broth.

Procedure.

All blood or clot culture flasks, which showed growth, were subcultured to blood agar plates and to dextrose beef infusion broth.

With throat cultures, as a rule, 8 suspicious colonies were picked and transferred to a blood agar plate. Those producing characteristic colonies in pure culture after 24 hours incubation were transferred to dextrose beef infusion broth. All strains proving to be streptococci were then transferred to 1.0 per cent lactose beef infusion broth containing phenol red and were also tested for agglutination with rabbit antisera prepared with representative strains of the Small and Birkhaug types of non-hemolytic streptococci.

Gamma type streptococci which differed only in their ability to ferment lactose were frequently recovered from the same throat. Representatives of both the lactose-fermenting and non-lactose-fermenting types were always saved. Duplicate strains were discarded. More extensive sugar fermentation tests and agglutination tests were eventually carried out. When warranted, agglutinin absorption tests were done. The agglutinin content of the patients' sera for autogenous and, sometimes, for various other strains was determined. All strains were tested for the production of a skin-reacting soluble toxin. A more detailed account of the methods follows:

Morphology.—Smears were made and stained by Gram's method from 24 to 48 hour cultures in dextrose broth.

Cultural Characteristics.—Notes were made concerning surface colonies on blood agar plates and on the type of growth in dextrose broth. All strains were transferred to tubes of beef infusion broth (pH 7.8) containing 1.0 per cent dextrose, lactose, saccharose, mannitol, salicin, inulin or raffinose, together with suitable amounts of phenol red; final readings as to fermentation were taken after 5 days incubation.¹

Agglutination.—Antisera were prepared by injecting rabbits intravenously at 4 or 5 day intervals with 24 or 48 hour cultures of the desired organisms in dextrose broth. The usual dosage was 1, 5, 10, 10, 10 and 10 cc. After an interval of 7 or 8 days, a test bleeding was taken, and, if a sufficiently high titer was obtained, the animal was bled. Otherwise, the injections were repeated.

For the test, 0.5 cc. of a 24 or 48 hour dextrose broth culture was added to an equal amount of the proper dilution of the antiserum. Many of the strains were tested with increasing dilutions starting with a final serum dilution of 1:80. The majority, however, were tested with a single dilution sufficiently high to rule out "group agglutination." Controls with normal salt solution and anti-*B. paratyphosus* B rabbit serum were always set up. The tubes were placed in a water

¹ It had been previously determined that the amount of sugar normally present in beef infusion broth was insufficient to bring about, by its fermentation, significant change of the indicator. This obviated the necessity of using sugar-free broth.

bath at $\pm 40^{\circ}\text{C}$. for 24 hours. One plus represented definite agglutination when viewed with a hand lens and 4 plus complete agglutination with a crystal-clear supernatant fluid.

Agglutinin Absorption.—48 hour cultures of the desired organisms in 50 cc. dextrose broth were centrifuged at about 3000 revolutions per minute for 40 minutes—no attempt was made to standardize the number of organisms except to discard any culture showing poor growth. The clear supernatant fluid was discarded and 5.0 cc. of a 1:160 dilution of the desired antiserum added. The tubes were incubated at 37.5°C . for 3 hours with frequent shaking and left at room temperature ($\pm 22^{\circ}\text{C}$.) for 21 hours. They were then centrifuged and the clear supernatant fluid pipetted away. The homologous organism, in 0.5 cc. amounts of a dextrose broth culture, was added to equal amounts of increasing dilutions of the absorbed antiserum. These tubes were subsequently treated like the agglutination tests. With each antiserum and with every series of tests, control tubes of unabsorbed serum and of serum absorbed with the homologous organism were always carried through.

Agglutinins in Patients' Sera.—Increasing dilutions of each patient's serum were prepared. Equal amounts (0.5 cc.) of all autogenous strains and various other representative strains were added from 24 to 48 hour dextrose broth cultures. The lowest final dilution of the serum was 1:40. Control tubes with normal salt solution and a non-agglutinating normal serum were made. All tubes were subsequently treated like the agglutination tests. Control tests with sera from non-rheumatic patients were carried out.

Toxin Production.—All strains were grown for 10 days in 50 cc. amounts of modified Douglas tryptic digest broth, as described by Birkhaug (3). The cultures were centrifuged and the supernatant fluid filtered through Berkefeld N or V candles. The filtrates were tubed and kept in the ice box. No phenol or tricresol was added. Sterility tests were always carried out. For testing, 1:10 dilutions were made with sterile salt solution and these were injected intradermally in 0.1 cc. amounts in an individual known to be skin-positive to similar amounts of Birkhaug's standard test toxin.²

RESULTS.

Stock Strains.—A number of strains of non-hemolytic streptococci from cases of rheumatic fever were obtained through the kindness of Dr. James C. Small of Philadelphia and Dr. Konrad E. Birkhaug of Rochester, New York. The 2 strains obtained from the former were labeled "A" and "B," and from now on will be referred to as "Small A" and "Small B."³ The 7 strains from the latter were labeled "1b,"

² This toxin (1:100 dilution, Lot 3, July 5, 1927) was obtained through the kindness of Dr. Konrad E. Birkhaug of Rochester, New York.

³ "Small A" and "Small B" correspond to "R1" and "R9" in Small's original paper (2).

"2," "10a," "17," "36," "84" and "167," and will be referred to as "Birk. 1b," "Birk. 2" and so forth.

Morphologically and culturally these 9 strains were practically identical. In broth they all produced a rather heavy ropy sediment with a relatively turbid supernatant fluid. On smear they appeared as rather small Gram-positive diplococci with numerous short to moderately long chains. On blood agar the colonies were round (maximum 2.0 to 3.0 mm. in diameter), tent-shaped, soft, moist, translucent and grayish in color, and they were readily picked out in a mixed culture by a peculiar reddish brown color apparent with transmitted light. There was no change in the color of the surrounding medium except a rather diffuse paling, which occurred after a number of days at ice box temperature. All strains fermented dextrose, saccharose, lactose, raffinose, salicin and inulin, but failed to ferment mannitol.

A number of rabbits were injected intravenously with varying amounts of 24 hour cultures of certain of these streptococci in dextrose broth. For producing monovalent agglutinating sera 3 rabbits were injected with living cultures of Small A, Small B and Birk. 2, respectively. They received injections of 1, 5, 10, 10, 10 and 10 cc. at 4 to 5 day intervals. All 3 failed to show any rise in temperature and the loss in weight was negligible. The first 2 were killed 10 days after the last injection. One (Small A), at autopsy, was negative except for the lungs, which were distended, firm and cut with increased resistance. The other (Small B) showed identical pulmonary changes and, in addition, several firm to friable vegetations on the wall of the left auricle and on the papillary muscles of the tricuspid valve. The third rabbit (Birk. 2) died 2 days after the last injection. The autopsy was negative except for pulmonary changes like those present in the other 2 rabbits.

Two rabbits were given larger amounts of 2 of the cultures—Small A, 10, 20, 18, 20 and 18 cc. and Small B, 10 and 20 cc. at 4 to 5 day intervals. The Small A rabbit was found dead the morning following the last injection. At autopsy, there were friable vegetations on the papillary muscles of the tricuspid valve. The lungs were enlarged and firm. Cultures taken of these vegetations and the heart's blood yielded pure cultures of non-hemolytic streptococci. The Small B

rabbit died 19 hours after the last injection. The autopsy and cultures of the heart's blood were negative. Two more animals received still larger doses—Small A, 23 and 50 cc. and Small B, 23 and 50 cc. In each instance the desired amount of culture was centrifuged at about 3000 revolutions per minute for 30 to 40 minutes, and the sediment resuspended in about 10 cc. of sterile salt solution. The second injections were given the 2nd day following the first. The Small A rabbit was found dead the morning following the second injection. The autopsy was negative, but the blood cultures were positive. The Small B rabbit died on the 5th day following the second injection; the autopsy and blood culture were negative. Two more rabbits received larger doses of heat-killed (100°C. for 1 hour) cultures—Small A, 50 and 50 cc. and Small B, 50 and 50 cc. These were centrifuged and the organisms resuspended in 10 cc. of sterile salt solution. The second injections were given on the 4th day following the first. Both animals gained in weight and failed to show any harmful effects.

Careful inspection of all leg joints, both during life and at autopsy, failed to show the slightest evidence of any sort of joint lesion in any of the animals. The consistent pulmonary changes in animals receiving repeated injections were found to be secondary to a blocking of the veins and capillaries by subendothelial collections of large mononuclear cells. Identical lesions were produced by heat-killed non-hemolytic (gamma type) streptococci and also by certain strains of hemolytic streptococci. The changes apparently represented a peculiar reaction to certain bacterial constituents and will be reported in a subsequent paper.

A group of 50 adult persons was injected intradermally with 0.1 cc. amounts of Birkhaug's standard test toxin. Of the 35 without histories of rheumatic fever, chorea or persistently recurring "sore throats," 12 (34 per cent) gave positive skin reactions; of the 9 without histories of rheumatic fever or chorea, but with histories of "many sore throats," 2 (22 per cent) were positive; and, of the 6 with histories of rheumatic fever, 2 (33 per cent) were positive. Thirteen children convalescent from acute rheumatic fever and with definite damage to one or more valves of their hearts were tested at the House of the Good Samaritan, Boston, through the kind cooperation of the Medical Staff. Of these, 8 (62 per cent) gave positive skin reactions. Control

TABLE I.

Summary of Results from Cultures from Patients with Acute Rheumatic Fever and from Normal Individuals.

Case No.	Rheumatic fever									Controls				
	Age and sex	Blood cultures					Stool culture	Urine culture	Throat culture	Clinical diagnosis	History			Throat culture
		Broth		Blood agar plates	Clot						R.F. or chorea	Sore throat	Tonsillectomy	
		No. 1	No. 2		No. 1	No. 2								
1	yrs. 19 ♂	C-10	—	0	0	0	0	0	+	? gastric path.	0	0	0	+
2	25 ♂	0	—	0	0	—	0	0	0	Cancer of breast	0	0	0	+
3	14 ♂	0	—	0	0	0	—	0	0	Normal	0	+	0	+
4	41 ♂	0	—	0	0	0	0	0	+	Normal	0	++	+	+
5	45 ♀	0	—	0	0	0	—	—	0	Normal	0	++	+	+
6	27 ♂	0	—	0	0	0	0	0	+	Diabetes	0	+	0	0
7	29 ♀	0	—	0	0	0	—	—	+	Normal	0	+++	+	+
8	28 ♀	0	—	0	0	0	—	—	+	Normal	0	0	0	0
9	12 ♀	C-34	0	0	0	—	—	—	+	Normal	0	+	+	+
10	27 ♂	0	0	0	0	—	—	—	+	Normal	0	0	0	+
11	31 ♀	0	0	0	C-30	—	—	—	+	Normal	0	++	+	+
12	34 ♀	0	0	0	0	—	—	—	+	Normal	0	++	+	+
13	17 ♂	0	0	0	C-23	C-23	—	—	+	Normal	0	+	0	+
14	25 ♀	C-36	0	0	0	—	—	—	+	Normal	0	+	+	0
15	19 ♀	0	0	0	0	—	—	—	+	Normal	0	+	0	+
16	35 ♂	0	0	0	0	—	—	—	+	Normal	0	+	+	+
17	17 ♂	C-5	0	0	C-12	—	—	—	+	Normal	0	0	0	+
18	23 ♂	0	0	0	0	—	—	—	+	Normal	0	+	0	+
19	20 ♂	0	0	0	0	—	—	—	+	Normal	0	0	0	+
20	21 ♂	C-20	0	0	0	—	—	—	0	Normal	0	+	0	+
21	20 ♀	0	0	0	0	—	—	—	+	Normal	0	++	+	+
22	16 ♀	0	0	0	0	—	—	—	0	Normal	0	0	0	+
23	39 ♂	0	0	0	0	—	—	—	+	Normal	0	+++	+	+
24	16 ♀	0	0	0	0	—	—	—	+	Normal	0	0	0	+
25	14 ♂	0	0	0	0	—	—	—	+	Normal	0	0	0	+

0 = no growth under cultures, otherwise absent.

C = contaminated—numbers represent age of culture in days.

+ = gamma type streptococci under cultures, otherwise present.

tests with heated toxin (100°C. for 2 hours) gave results identical with those of the unheated toxin.

Cultures.—The results of the various cultures are given in Table I. The blood from each case of acute rheumatic fever was cultured in various ways and no streptococci of any sort were ever recovered.

Organisms were recovered from the broth cultures in 5 cases, as follows:

Case 1.—Staphylococci were found on the 10th day. There was no duplicate culture. The clot cultures and blood agar plates were negative.

Case 9.—Gram-positive bacilli were found on the 24th day in 1 of the flasks. The duplicate culture, clot culture and blood agar plates were negative.

Case 14.—*Staphylococcus albus* was found on the 26th day in 1 of the flasks. The duplicate culture, clot culture and blood agar plates were negative.

Case 17.—Gram-positive bacilli were found on the 5th day in 1 of the flasks. The duplicate culture and blood agar plates were negative, but the clot culture showed Gram-positive bacilli on the 12th day.

Case 20.—Staphylococci were found on the 20th day in 1 of the flasks. The duplicate culture, clot culture and blood agar plates were negative.

Organisms were recovered from the clot cultures in 3 cases, as follows:

Case 11.—*Staphylococcus albus* was found on the 30th day. The broth cultures and blood agar plates were negative.

Case 13.—*Staphylococcus albus* was found on the 23rd day in both cultures. The broth cultures and blood agar plates were negative.

Case 17.—As above.

The blood agar plates were consistently negative.

Stool and urine cultures were made in 5 out of the first 6 cases. As no colonies were obtained which were suggestive of non-hemolytic streptococci, they were not made from the other cases.

The findings on the blood agar plates from throat swabs were quite different from the negative blood cultures. In the first 5 cases, organisms were recovered from 2 which could not be distinguished, either morphologically or culturally, from the Small and Birkhaug strains. In 18 of the remaining 20 cases, similar organisms were recovered; and it seems likely that they would have been recovered in a higher percentage of the first 5 cases, if, at that time, the characteristic appearance of the colonies on blood agar had been better known.

Throat cultures were made from an equal number of individuals who gave no history of rheumatic fever, chorea or heart disease. By far the majority were from members of the laboratory staff. Non-hemolytic streptococci of the gamma type were recovered from 22 cases (88 per cent), as compared with 20 cases (80 per cent) in the acute rheumatic fever series.

Serological Tests.—A number of rabbits were immunized against certain strains of these non-hemolytic (gamma type) streptococci (Small A and B, Birk. 2, RFT 4 and NT 2-8 and 5-2). All of the stock strains and those obtained from rheumatic fever and normal throats were tested, if possible, against all 6 of these antisera. A number of the strains agglutinated spontaneously in salt solution, and, although various media, such as plain broth and phosphate-buffered broth, were used, satisfactory growth could not be obtained.

Preliminary tests showed the presence of a common antigenic base in all strains, for, in a series of increasing dilutions, practically all organisms tested showed complete agglutination as high at 1:160 with all antisera. As the dilutions were carried higher, however, 2 rather distinct groups became apparent—the one represented by Small A and B and the other by all but 1 (Birk. 36) of the Birkhaug strains. The latter group is apparently identical with the group designated by Hitchcock (5) as "Type I." There were a certain number of strains which did not fall into either of these 2 groups; some failed to show any significant agglutination and others were agglutinated by all antisera, but not by the control serum or salt solution.

In doing the routine agglutination tests on all strains the dilutions of the various antisera were such that "group agglutination" was excluded. The results are shown in Table II.

Each strain has been assigned to 1 of 5 groups—Small, Birkhaug, non-agglutinating, agglutinating or spontaneously agglutinating. This division is, in many instances, far from clear-cut, but serves for the purpose of demonstrating the lack of homogeneity among these gamma type streptococci.

Three antisera were prepared from throat strains isolated during the course of this investigation. One (RFT 4) belongs, clearly, to the Birkhaug group, another (NT 2-8) to the agglutinating group and the other (NT 5-2), questionably, to the Small group. It is most interesting to note that, although the strains belonging to the agglutinating group are agglutinated by practically all of the antisera, the antiserum (NT 2-8) of this group fails to agglutinate any strains, in an appreciable amount, other than those belonging to the group.

A résumé of the different groups recovered from the cultures of the normal and rheumatic fever throats is given in Table III. Birkhaug

TABLE II.
Serological and Cultural Characteristics of Stock and Isolated Strains of Gamma Type Streptococci.

Strain	Agglutination tests								Agglutinin absorption		Lactose fermentation	Toxin production	Serological group	Remarks
	Salt solution control	Anti- <i>B. paratyph. B</i> control 1:640	Anti-Small A 1:1280	Anti-Small B 1:1280	Anti-Birk. 2 1:1280	Anti-RF 4, 1:2560	Anti-NT 2-8, 1:2560	Anti-NT 5-2, 1:1280	Anti-Small A	Anti-Birk. 2				
Small A	0	0	++	++	++	++	++	++	++	++	++	++	S	
B	0	0	++	++	++	++	++	++	++	++	++	++	S	
Birk. 1b	0	0	0	0	++	++	++	++	++	++	++	++	B	
2	0	0	0	0	++	++	++	++	++	++	++	++	B	
10a	0	0	0	0	++	++	++	++	++	++	++	++	B	
17	0	0	0	0	++	++	++	++	++	++	++	++	B	
36	0	0	0	0	++	++	++	++	++	0	++	++	S	
84	0	0	0	0	++	++	++	++	++	++	++	++	B	
167	0	0	0	0	++	++	++	++	++	++	++	++	B	
RFT 1	0	0	0	0	++	++	++	++	++	++	++	++	B	
4	0	0	0	0	++	++	++	++	++	++	++	++	B	
6	++	0	0	0	++	++	++	++	++	++	++	++	SA	
7	0	0	0	0	++	++	++	++	++	++	++	++	B	
8-2	++	0	0	0	++	++	++	++	++	++	++	++	SA	
5	0	0	0	0	++	++	++	++	++	++	++	++	B	
9-1	++	0	0	0	++	++	++	++	++	++	++	++	SA	
13	++	0	0	0	++	++	++	++	++	++	++	++	SA	
10-3	0	0	0	0	++	++	++	++	++	++	++	++	A	
5	0	0	0	0	++	++	++	++	++	++	++	++	B	

	1											2											
	B	B	B	NA	B	-	B	B	SA	SA	SA	B	B	B	SA	B	S	B	SA	B	B	SA	A
11-6	0	0	0	+	0	+	0	+	0	0	+	0	0	0	+	0	0	0	+	0	0	0	0
8	+	+	+	+	+	0	+	0	+	+	+	+	+	0	+	+	0	+	+	+	+	0	+
12-3	0	+	+	0	+	+	0	0	0	0	0	0	0	0	+	+	+	+	+	+	+	+	+
7	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
13-1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
14-7	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
15-6	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
7	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
16-8	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
17-1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
18-2	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
3	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
19-1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
3	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
21-3	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
23-1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
24-1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
4	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
5	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
6	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
25-2	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
6	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
7	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0

Agglutinin adsorption: ++++ = complete, +++ = marked, ++ = moderate, + = slight.

Serological groups: S = Småll, B = Birkhaug, A = agglutinating, SA = spontaneously agglutinating, and NA = non-agglutinating.

Remarks: 1 = growth unsatisfactory for agglutination, 2 = raffinose not fermented, and 3 = grown in phosphate-buffered broth for agglutination tests.

TABLE II—Concluded.

Strain	Agglutination tests								Agglutinin absorption		Lactose fermentation	Toxin production	Serological group	Remarks
	Salt solution control	Anti- <i>B. paratyph. B</i> control 1:640	Anti-Small A 1:1280	Anti-Small B 1:1280	Anti-Disk. 2 1:1280	Anti-RF 4, 1:2560	Anti-NT 2-8, 1:2560	Anti-NT 5-2, 1:1280	Anti-Small A	Anti-Disk. 2				
NT 1-1	0	0	0	0	++	++	++	0		++	++	0	NA	3
2-6	0	0	0	0	++	++	++	0	+	++	++	0	B	
8	0	0	++	++	++	++	++	++	++	++	++	0	A	
3-1	0	0	++	++	++	++	++	++	++	++	++	0	S	
3	0	0	0	0	++	++	++	0		++	++	0	B	
4-4	0	0	++	++	++	++	++	++		++	++	0	B	
5-1	0	0	++	++	++	++	++	++	+	++	++	0	A	
2	0	0	0	0	++	++	++	++	+	++	++	0	S	
7-2	0	0	0	0	++	++	++	++		++	++	0	B	
4	0	0	++	++	++	++	++	++		++	++	0	S	
9-2	0	0	++	++	++	++	++	++		++	++	0	A	
3	++	0	+	+	++	++	++	++		++	++	+	SA	
10-1	++	0	0	0	++	++	++	0		++	++	0	B	
3	++	0	0	0	++	++	++	0		++	++	0	SA	
11-2	0	0	0	0	++	++	++	0		++	++	0	B	
12-6	0	0	0	0	++	++	++	0		++	++	0	NA	
13-3	0	0	0	0	++	++	++	0		++	++	0	B	
15-4	0	0	0	0	++	++	++	0		++	++	0	S	
7	0	0	0	0	++	++	++	0		++	++	0	B	
16	0	0	0	0	++	++	++	0		++	++	+	B	

[illegible]

strains (Hitchcock (5)—Type I) were recovered in approximately equal numbers in each of the 2 groups. Many more Small strains were obtained from the normal throats, whereas the other groups occurred with about the same frequency.

Agglutinin absorption tests were carried out with a number of the strains. The results are included in Table II. Many strains that were agglutinated in as high a dilution by the antiserum as the homologous strain failed to show even the slightest degree of absorption of agglutinin. This was particularly true of the strains belonging to the Birkhaug group—for example, from the same throat 1 strain (RFT

TABLE III.

Summary of the Occurrence of the Various Kinds of Gamma Type Streptococci in Throats of Patients with Acute Rheumatic Fever and of Normal Individuals.

Variety of gamma type streptococci	Rheumatic fever throats				Normal throats			
	Occurrence		Percentage occurrence		Occurrence		Percentage occurrence	
	All cases	Alone	All cases	Alone	All cases	Alone	All cases	Alone
Birkhaug.....	14	8	56	32	15	7	60	28
Small.....	1	0	4	—	8	—	32	—
Non-agglutinating.....	1	0	4	—	2	2	8	8
Agglutinating.....	2	0	8	—	3	—	12	—
Spontaneously agglutinating.....	9	5	36	20	6	1	24	4
None.....	5	—	20	—	3	—	12	—

11-8) completely absorbed the Birk. 2 antiserum, while the other (RFT 11-6) failed to show any absorption.

All but 1 of the rheumatic fever patients' sera and a number of sera from individuals who gave no history of rheumatic fever were tested for agglutinins against representative strains of the Small and Birkhaug groups. The former sera were also tested, whenever possible, against their own throat strains. Agglutination was considered significant only in the presence of a 3 plus agglutination in a final serum dilution of 1:40, or a 2 plus in this dilution, which was repeated in the 1:80 dilution. In neither the rheumatic fever nor the control sera could agglutinins be demonstrated for Small A or Birk. 2. Five

(Cases 1, 4, 5, 7 and 18) out of 22 rheumatic fever sera and 1 (Case 13) out of 14 control sera agglutinated RFT 4. Three (Cases 9, 11 and 18) out of 17 rheumatic fever sera and 2 (Cases 1 and 3) out of 17 control sera showed agglutinins for RFT 8-5; and 1 (Case 11) out of 17 rheumatic fever sera and 1 (Case 8) out of 17 control sera showed agglutinins for NT 16. Four (Cases 4, 11, 12 and 25) out of 15 rheumatic fever sera agglutinated 1 of the autogenous strains. The only difference noted between the two series was the greater ability of the rheumatic fever sera to agglutinate RFT 4. As this particular strain was frequently spontaneously agglutinated and, hence, gave proof of instability, too much significance should not be attached to the observation.

Toxin Production.—All strains isolated from the rheumatic fever and normal throats were tested for the production of a soluble skin-reacting toxin. The tests were carried out in the skin of an individual known to react strongly to Birkhaug's standard test toxin. The results of these skin tests are recorded in Table II. Of the rheumatic fever strains, 7 gave positive skin reactions, but only 3 were of sufficient intensity (erythema 1.0 cm. or more in diameter) to be significant in a dilution of the filtrate as low as 1:10. Of the 40 normal throat strains, 4 were positive, 2 being of moderate intensity.

In order to make certain of the identity of these skin reactions with those of Birkhaug, 37 adult individuals were injected, intradermally and at the same time, with 1 (Birk. 1b, 1:10) of these toxins and with Birkhaug's standard toxin. In 33 instances the skin reactions to both toxins were identical (9 positive and 24 negative), whereas in 4 they disagreed (Birk. 1b positive with standard toxin negative in 3 and standard toxin positive with Birk. 1b negative in 1).

DISCUSSION.

The comparison of the Small and Birkhaug strains of non-hemolytic streptococci would seem to indicate that, although they are very closely related, they are not identical. This finding agrees with the observations of Hitchcock (5).

There are two outstanding discrepancies when the experimental findings reported herein are compared with those of Small and Birkhaug. In the first place, no joint changes, such as those described by

Small (2), were ever observed in the rabbits which had received repeated large doses of living organisms. It is true that fresh vegetations on the valves and papillary muscles of the heart were observed on 2 occasions, but this might be expected following the intravenous injection of almost any living organism in such large numbers. Furthermore, it should be borne in mind that rabbits with a streptococcus septicemia, regardless of the source or variety of the streptococci, frequently show acute joint involvement along with other localized foci of infection. Secondly, it has been impossible to demonstrate that the majority of these strains of streptococci produce a soluble skin-reacting toxin. Birkhaug (3) states that 72 per cent of his non-methemoglobin-producing streptococci gave strongly positive skin reactions in dilutions as high as 1:100. Such a toxin was found in only 1 (Birk. 1b) of Birkhaug's 7 strains, and dilutions as low as 1:10 of the other 6 failed to give definite skin reactions. Although no phenol was added before filtration and although the filtrates were tested oftentimes within a few days after filtration, rather than after 1 month in the ice box, as Birkhaug did, it is difficult to conceive of either procedure as being capable of influencing the final result. As previously stated, the 1 toxic filtrate (Birk. 1b) gave practically identical skin reactions as the Birkhaug standard test toxin, when the tests were carried out simultaneously.

Non-hemolytic streptococci of the gamma type were never recovered in the blood cultures from 25 cases of acute rheumatic fever. Bacteria were occasionally isolated, but they were never found consistently in all cultures. Those recovered were of the type to be expected as a result of contamination, and they were usually found only after 10 or more days incubation. From the papers of Small (2) and Birkhaug (3) it would appear that the former obtained his organisms in 2 out of 31 cases and the latter in 2 out of 36. Hitchcock (5) also obtained 2 strains from blood cultures of patients with rheumatic fever. It must be acknowledged that the present series of 25 cases is small and different results might obtain in a larger series. The blood cultures also failed to reveal the presence of streptococci of the alpha type, such as those described by Clawson (1).

From the throats of 20 out of 25 cases of acute rheumatic fever non-hemolytic streptococci were recovered which were identical,

morphologically and culturally, with the Small and Birkhaug strains. As judged by agglutination, the majority of strains were similar to those of the latter. As has been previously mentioned, the Birkhaug strains apparently correspond to the Type I of Hitchcock (5). Agglutinin absorption tests with these Birkhaug similar strains would seem to indicate that 3 out of the 18 strains were identical with Birk. 2. There were many strains, however, which, although they were agglutinated by anti-Birk. 2 serum in high dilution, showed a complete absence of ability to absorb the specific agglutinin from the same serum. Reciprocal agglutinin absorption tests were not carried out. When tested for toxin production, only 3 filtrates from the 34 strains were found which were able to bring about a definite skin reaction in a dilution as low as 1:10.

Similar non-hemolytic streptococci were recovered from 22 out of 25 normal throats. The only difference noted in this series, as compared with the strains isolated from rheumatic fever throats, was a considerably higher number of Small strains. The rather universal presence of these streptococci in both the rheumatic and non-rheumatic throats and the apparent predominance of Birkhaug similar strains, as determined by agglutination, agree very closely with the figures of Hitchcock (4).

From a comparison of these 2 series it would seem reasonable to conclude that non-hemolytic (gamma type) streptococci are of relatively common occurrence in the human throat. The fact that practically all strains are agglutinated by relatively low dilutions of antisera of various type strains argues for a common antigenic base. When the dilutions are carried higher, however, it becomes apparent that the strains are far from identical. Agglutinin absorption tests bring the heterogeneity of this group even more in evidence.

Sera from rheumatic fever patients have failed to show agglutinins for the Small or Birkhaug strains. It must be acknowledged that these sera were obtained during the acute stage of the disease and that such agglutinins might appear subsequent to improvement or recovery.

From the work reported here, very little can be said concerning the etiologic importance of these streptococci in relation to the conception of acute rheumatic fever as a manifestation of bacterial allergy

(Zinsser (6, 7), Swift, Derick and Hitchcock (8) and Birkhaug (9)). The findings, however, fail to indicate that such streptococci should be considered as the specific etiologic factor in acute rheumatic fever. Kaiser (10) has reported a large series of cases which have been tested for skin reactivity to a soluble toxin obtained from 1 of these streptococci and there can be no doubt but that children with a history of rheumatic fever or chorea give a higher percentage of positive reactions than children without such a history. There are, however, at least two facts which should be considered. In the first place, this toxin is extremely thermostabile, a fact which distinguishes it sharply from the true exotoxins, such as diphtheria and tetanus toxins, and makes it hardly comparable, even, to Dick toxin, which is relatively *thermostabile*. Secondly, *very little is known concerning skin reactivity*, in general, to non-specific foreign proteins during or following different diseases. The recent work of Irvine-Jones (11) brings out the fact that persons suffering from rheumatic fever, particularly during the acute stage, are relatively skin-sensitive to the filtrates from various sorts of streptococcic cultures. Whether or not such individuals are sensitive to the filtrates from cultures of other types of bacteria or to protein substances of primarily non-toxic nature remains to be demonstrated. Kaiser's observations certainly point to a specific relationship, but until more is known concerning the nature of such toxins and concerning the significance of skin reactivity in various diseases it hardly seems justifiable to draw any definite conclusions.

SUMMARY AND CONCLUSIONS.

1. Blood cultures from 25 cases of acute rheumatic fever were negative for non-hemolytic streptococci of both the alpha and gamma types.

2. Non-hemolytic (gamma type) streptococci were frequently recovered from the throats of patients with this disease.

3. Similar organisms were recovered just as frequently from the throats of normal individuals.

4. Although these non-hemolytic streptococci were morphologically and culturally identical, not only amongst themselves, but also when compared with stock Small and Birkhaug strains, all, including the latter, have failed to show any noteworthy degree of homogeneity.

5. Representative strains of these streptococci have proved to be relatively non-pathogenic for rabbits following intravenous injection.

6. These organisms, with a few exceptions, have failed to produce soluble skin-reacting toxins comparable to Birkhaug's standard test toxin.

7. The foregoing facts seem to invalidate the assumption that any of these non-hemolytic streptococci play a specific rôle in the etiology of acute rheumatic fever.

We wish to express our indebtedness to Miss Miriam MacKay for technical assistance.

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THE INITIATION OF GROWTH OF CERTAIN FACULTATIVE ANAEROBES AS RELATED TO OXIDATION-REDUCTION PROCESSES IN THE MEDIUM.

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The expression "value of a bacteriological medium" is a loose one; it probably covers a number of characteristics of that medium, many of which are perhaps unrelated. The following are examples of the points of view from which the medium may be considered in this relation.

1. Initiation of growth: (a) size of minimum inoculum required for growth to develop, (b) duration of the lag period.
2. Density of growth: number of cells developing per unit capacity of medium.
3. Viability of the cultures.

This paper will be limited to a survey of some of the conditions which affect the size of the inoculum required to initiate growth.

HISTORICAL.

It is a well known fact that the growth of organisms difficultly cultivable is rendered much easier—even in unfavorable media—by the use of large inocula. This phenomenon is probably related to what has been termed "allelotaxis" by Robertson (1) in his studies on transplants of protozoa, and "communal activity," by Churchman and Kahn (2) in their investigations on the bacteriostatic action of certain dyes.

The work of Valley and Rettger (3) indicates that many organisms grow slowly or not at all until a certain minimum concentration of carbon dioxide is present in the medium; the suggestion has been made that the introduction of a large inoculum may hasten the production of a favorable concentration of CO₂.

Gillespie (4) observed that "it requires much smaller numbers of pneumococci to start growth on agar than are required to start a growth in broth." He found also that the same result as with agar could be obtained by seeding the pneumococci on a fragment of filter paper on the surface of the broth. According to him, "this phenomenon is probably dependent entirely on physical differences

in the two kinds of media, and bears some relation to the differences in possibilities for diffusion in the two media."

The possible relation of minimum inoculum to oxidation-reduction processes has been expressed several times in the literature. In the course of his studies on the physiology of *B. lepusculum*, Webster (5) observed that an inoculum of at least 100,000 cells (per 5 cc. broth), was necessary for growth to develop under aerobic conditions. On the other hand, growth occurred with an inoculum of only a very few cells when the culture was incubated under anaerobic conditions, or in the presence of sterile blood.

Burnet (6) showed that nutrient agar plates, exposed to light, while still capable of growing staphylococcus when heavily seeded, did not allow growth of "isolated" organisms. ("Isolated" organisms were obtained by spreading over the plate a very dilute suspension of bacteria.) But such "isolated" organisms would grow in the neighborhood of colonies of the same or other organisms, obtained by local heavy seedings. Nutrient agar which had been exposed to light and then heated for 30 minutes at 100°C. gave normal growth, unless exposure to light had been extremely prolonged. The explanation offered was that exposure to light resulted in the formation of a peroxide-like substance with a bacteriostatic action; reducing substances, formed in the course of metabolism in the large colonies (obtained by local heavy seedings) diffused through the agar and reduced the peroxides, thus allowing growth of "isolated" organisms around the colonies. Destruction of the peroxide was also obtained by heating the medium. Working with an anaerobe, *B. sporogenes*, Quastel and Stephenson (7) observed that this organism could be grown under apparent "aerobic" conditions, in tryptic digested broth, by the use of a large inoculum. A very small inoculum was sufficient to initiate "aerobic growth" when 0.1 per cent cysteine was added to the broth. Reduced glutathione and thioglycollic acid were found to play the same rôle as cysteine. According to Quastel and Stephenson, the mechanism of the process is as follows: In the course of its metabolism, *B. sporogenes* produces reduced compounds giving the $-SH$ reaction; the object of a large inoculum is to introduce enough of these reduced sulphhydryl compounds to provide reducing conditions in the broth. The addition of cysteine serves of course the same purpose.

W. M. Clark (8) had previously pointed out that actively growing anaerobic cells, if not overtaxed, can establish their own reducing conditions even in the presence of a certain amount of molecular oxygen.

Aubel and Aubertin (9) seeded different organisms into tubes of glucose agar and observed at what levels of the agar growth developed. By observing the reduction of indicators of oxidation-reduction potentials in sterile tubes of the same medium, and comparing the findings with the results of the growth experiments, they concluded that the life of strict anaerobes is possible only when the rH of the medium is lower than 12.

Before attempting to analyze the mechanism whereby the oxidation-reduction properties of the medium control the minimum inoculum required to initiate growth, it is important to realize that bacterial cells themselves possess active and independent oxidation-reduction systems. The manifestations and nature of these systems are especially well known in the case of *Pneumococcus*.

Avery and Neill (10) have described a number of different oxidation-reduction processes which are exhibited by cultures or sterile extracts of pneumococci; such are the consumption of molecular oxygen, the production of peroxide, the oxidation of hemotoxin, the oxidation of hemoglobin to methemoglobin, and the oxidative destruction of various endocellular enzymes. The same cultures or extracts of pneumococci also reduce methylene blue to methylene white and methemoglobin to hemoglobin when the system is kept under anaerobic conditions.

These active oxidation-reduction systems of the cells consist of two components: (1) a cellular thermolabile constituent which is not removed by washing, (2) thermostable autoxidizable substances which are lacking in washed cells and which are not necessarily of pneumococcus origin, since they may be supplied by muscle infusion and yeast extract.

It is probable that a complete understanding of the influence of oxidation-reduction processes in bacterial growth will require a study of the interrelations between the oxidation-reduction systems of the media and of the cells.

EXPERIMENTAL.

The influence of the nature of the medium and of environmental conditions on the size of the minimum inoculum giving rise to growth, was studied by the following technique.

Tubes containing 5 cc. of the medium under consideration were inoculated with varying amount of culture. These inocula are referred to as 10^{-1} , 10^{-2} , 10^{-3} , 10^{-4} , 10^{-8} , corresponding to 0.1....0.00000001 cc. of a 12 hour culture in plain broth.

The cultures used were: *Pneumococcus* S forms, Type I (1/200/5), II (D/39/40), III (A/66/64), and R forms derived from Types I (1/193/R), II (D/39/R), and III (M/3/R). Human strains of hemolytic streptococcus, L, S/43, S/23/Glossy, S/23/45 Matt. *Staphylococcus aureus*.

Inocula were never taken from blood cultures, but from the third transfer in plain broth in order to prevent the carrying over of some of the blood constituents into the medium to be tested.

These plain broth cultures were plated on blood agar to determine the number of organisms present in the inoculum. There was of course some variation, but in general, plates inoculated with 10^{-7} of plain broth cultures of *Pneumococcus* or human strains of *Streptococcus hemolyticus* showed only a few colonies (1 to 10). With *Staphylococcus*, the last positive plates were obtained with 10^{-9} cc. inoculum.

The plain broth was prepared from meat infusion according to the standard method. Fairchild's peptone (lot 280630) was used in the preparation. Cysteine solutions were prepared from cysteine hydrochloride and autoclaved. As the neutralized solution oxidizes very rapidly, while the acid solution is more stable, the latter was neutralized with sterile NaOH only at the time of being used. "Anaerobic" conditions were provided by sealing the cultures with a 2 cm. layer of sterile vaseline.

TABLE I.

Growth of Pneumococcus and Streptococcus in the Presence of Different Concentrations of Blood.

Medium	Smallest inocula with which growth was obtained			
	Pneumococcus II		<i>S. hemolyticus</i> (human strains)	
	"S" D/39/40	"R" D/39/R	L	S/43
Plain broth.....	10^{-2}	10^{-2}	10^{-2}	10^{-3}
Plain broth + 0.001 per cent blood.....	10^{-2}	10^{-2}	10^{-2}	10^{-2}
Plain broth + 0.01 per cent blood.....	10^{-4}	10^{-4}	10^{-5}	10^{-4}
Plain broth + 0.1 per cent blood.....	10^{-8}	10^{-8}	10^{-8}	10^{-7}
Plain broth + 1 per cent blood.....	10^{-7}	10^{-6}	10^{-6}	10^{-7}

I. Growth of Streptococcus and Pneumococcus in Blood Broth.

Blood broth is known to be an ideal medium for the growth of pneumococci and streptococci. The attempt was made to ascertain what concentration of blood is necessary to insure growth with the smallest possible inocula.

Experiment 1.—The plain broth used had been prepared 1 week before the test. Sterile rabbit blood was added to the medium in amounts sufficient to give final concentrations varying from 1 per cent to 0.001 per cent. Table I indicates, in cubic centimeters of plain broth cultures, the smallest inocula with which growth was obtained.

According to this experiment, blood is still active in a dilution of 0.01 per cent. It may be doubted whether the action of such a small amount

corresponds to the addition of some nutrient lacking in the broth. There have been suggestions that the action of the blood is due to the fact that it contains a peroxidase or a catalase and perhaps also the V factor which has been associated with yeast extract. It may be recalled here that Thjotta and Avery (11) found that two factors (X and V) are essential for the growth of certain strains of influenza bacillus. The factor V appears to be of the nature of a vitamine and can be supplied in the form of yeast extract; X was claimed to be an iron compound and hematin was used as its source in routine technique. X exhibits peroxidase and catalase activity. Both X and V are present in the blood.

Attempts have been made therefore to simulate the action of blood by the addition of certain iron compounds (possessing catalase, peroxidase, and oxidase activity) in the presence or in the absence of yeast extract.

II. The Influence of Iron Compounds on the Growth of *Pneumococcus*.

Experiment 2.—In these tests, a large number of iron compounds were used.¹ Among them may be mentioned.

Active Fe_2O_3 (Siderac)	} which exhibit both peroxidase and catalase activity.
Active Fe_2O_3 (Baudisch)	

Inactive Fe_2O_3 (Siderac)	} which exhibit only catalase activity.
Inactive Fe_2O_3 (Baudisch)	

Sodium pentacyano aquo-ferro salt	} which exhibit both catalase and peroxidase activity.
Sodium pentacyano aquo-ferri salt	
Sodium pentacyano amino-ferri salt	

In order to obtain comparative results, the following technique was used. The oxides were added to test-tubes in amounts of 100 to 500 mg. and autoclaved. 5 cc. of sterile broth were added later under sterile conditions. As to the ferro-aquo, ferri-aquo, and ferri-amino salts which are soluble and heat-labile, their solutions were filtered through Berkefeld filters and the filtrates added under aseptic conditions to sterile broth, the final concentrations varying from 1/1,000 to 1/1,000,000.

The tubes were inoculated with 8 hour cultures (in plain broth) of the different types of *Pneumococcus*. The results may be summarized as follows:

¹ All the compounds were obtained through the courtesy of Dr. Baudisch.

It is apparent that in no case was the plain broth improved by the addition of iron compounds. On the contrary, some of them seem to have a toxic action; such are the Siderac oxides and the ferri-amino salt.

The results were not changed when yeast extract was added to these media.

It is apparent that these iron compounds with or without the addition of yeast extract, do not give to plain broth those growth-promoting properties which are supplied by blood.

TABLE II.

Influence of Iron Compounds on the Growth of Pneumococcus (II R).

Medium	Inoculum (in cc.)					
	10 ⁻¹	10 ⁻²	10 ⁻³	10 ⁻⁴	10 ⁻⁵	10 ⁻⁶
Plain broth.....	+	+	-*	-	-	-
Blood broth.....	+	+	+	+	+	+
Plain broth + active Fe ₂ O ₃ (Siderac).....	-	-	-	-	-	-
Plain broth + active Fe ₂ O ₃ (Baudisch).....	+	+	-	-	-	-
Plain broth + inactive Fe ₂ O ₃ (Siderac).....	-	-	-	-	-	-
Plain broth + inactive Fe ₂ O ₃ (Baudisch)....	+	+	-	-	-	-
Plain broth + ferro-aquo salt.....	+	+	-	-	-	-
Plain broth + ferri-aquo salt.....	+	+	-	-	-	-
Plain broth + ferro-amino salt.....	+	-	-	-	-	-

* In the presentation of all these results, + or - indicates that growth did or did not develop.

III. Variations of the Growth-Promoting Properties of Plain Broth with Aging of This Medium.

In the course of these investigations, it has been repeatedly observed that when plain broth has been recently autoclaved, it allows the growth of very minute inocula of *Pneumococcus*. In order to establish this phenomenon more definitely, the following experiment was carried out.

Experiment 3.—Tubes containing 5 cc. of plain broth (1 week old), were autoclaved for 15 minutes, cooled down, and immediately inoculated with varying amounts of pneumococcus culture (D/39/R). Other tubes of the same autoclaved broth were kept for different intervals of time before being inoculated. Table III shows the highest dilution of inoculum with which growth was obtained at the different periods.

Similar results were obtained with other strains of *Pneumococcus* (Types I, II, III, virulent and avirulent) and with human strains of hemolytic streptococcus. Boiling the broth for 1 hour had the same effect as autoclaving.

With *Staphylococcus*, growth in "unboiled" broth (3 weeks old) was obtained up to 10^{-5} cc. inoculum, while it took place up to 10^{-8} in recently autoclaved broth.

TABLE III.

Growth of Pneumococcus (D/39/R) in Broth Inoculated at Different Times after Autoclaving.

Medium	Inoculum (in cc.)						
	10^{-1}	10^{-2}	10^{-3}	10^{-4}	10^{-5}	10^{-6}	10^{-7}
"Unautoclaved" broth ..	+	+	-	-	-	-	-
Broth inoculated immediately after autoclaving ..	+	+	+	+	+	+	+
Broth inoculated 2 hrs. after autoclaving....	+	+	+	+	+	+	+
Broth inoculated 4 hrs. after autoclaving ..	+	+	+	+	+	-	-
Broth inoculated 8 hrs. after autoclaving .	+	+	+	+	-	-	-
Broth inoculated 12 hrs after autoclaving	+	+	+	-	-	-	-
Broth inoculated 24 hrs after autoclaving.	+	+	-	-	-	-	-
Broth inoculated 48 hrs after autoclaving .	+	+	-	-	-	-	-
Broth inoculated 1 week after autoclaving .	+	+	-	-	-	-	-
Broth inoculated 3 weeks after autoclaving .	+	-	-	-	-	-	-

It is very important to point out that the effects of boiling or autoclaving are not limited to a short time after the treatment, but extend over 12 hours at least. As has been pointed out elsewhere (12) this indicates that the effect of the treatment is not limited to a mechanical removal of oxygen.

It has already been suggested that the effect of autoclaving or boiling is due to a breaking down or a reduction of oxidized substances

formed by the autoxidation of some constituents of the broth. If this is the case, similar results should be obtained by chemical methods of reduction. Experiments 4, 5, and 6 are examples of such methods.

Experiment 4.—A lot of broth (2 weeks old) was divided into 2 portions, 1 of which was reduced by hydrogen in the presence of palladinized asbestos, the asbestos being later filtered out in a nitrogen atmosphere. The untreated broth was used as control.

TABLE IV.

Growth of Pneumococcus (D/39/R) in Broth Reduced by Hydrogen.

Medium	Inoculum (in cc.)						
	10 ⁻¹	10 ⁻²	10 ⁻³	10 ⁻⁴	10 ⁻⁵	10 ⁻⁶	10 ⁻⁷
Plain broth.....	+	+	-	-	-	-	-
Reduced broth.....	+	+	+	+	+	+	+

TABLE V.

Influence of Vaseline Seal on the Growth of Different Organisms in Plain Broth.

Organism	Smallest inocula with which growth developed	
	Aerobic cultures	Sealed cultures
Pneumococcus D/39/R.....	10 ⁻²	10 ⁻⁴
A/66/64.....	10 ⁻¹	10 ⁻⁴
<i>S. haemolyticus</i> (L).....	10 ⁻²	10 ⁻⁴
S/43.....	10 ⁻¹	10 ⁻³
<i>Staphylococcus aureus</i>	10 ⁻⁵	10 ⁻⁸

The 2 portions were transferred to test-tubes (5 cc. per tube), and their growth-promoting power compared (see Table IV).

It appears that reducing the broth by hydrogen in the presence of palladinized asbestos affects its value in the same manner as boiling or autoclaving.

It has been indicated elsewhere (12) that plain broth kept under vaseline seal develops a reduction potential corresponding to reduced indigo disulfonate. In view of the results of Experiment 4, it was interesting to test whether incubation under vaseline seal would affect the size of the inoculum required to initiate growth.

Experiment 5.—Test-tubes containing 5 cc. of broth (2 weeks old) were inoculated with *Pneumococcus*, human strains of hemolytic streptococcus, and *Staphylococcus aureus*; some of the tubes were sealed with vaseline immediately after inoculation, while the others were incubated under aerobic conditions. Table V indicates the smallest inocula with which growth developed.

This experiment, the results of which have been repeatedly confirmed, show that initiation of growth is facilitated under vaseline seal. However, the results obtained were not so striking as the ones obtained with "boiled" or "autoclaved" broth, or, as shown later, by the addition of cysteine or ascitic fluid to the broth.

Sterile blood was added to the tubes which had failed to grow, but no growth developed on further incubation, thus indicating that the inoculum was dead. In the case of *Pneumococcus*, autolysis of the cells may account for the fact, but this explanation does not hold for *Streptococcus*; the significance of these observations will be discussed later.

IV. Growth of Pneumococcus, Streptococcus hæmolyticus, and Staphylococcus in Cysteine Broth.

Cysteine is an active reducing agent, capable of reducing indigo carmine (13). Its action in facilitating the growth of certain anaerobes has been reported and Quastel and Stephenson (7) suggested that, in the case of *B. sporogenes*, the beneficial action of cysteine was due to the establishment in the medium of a favorable reduction potential.

A preliminary experiment showed that, in the presence of 0.1 per cent cysteine, growth of *Pneumococcus* (D/39/R), human strains of *S. hæmolyticus* (L), and *Staphylococcus aureus* could be obtained with inocula of 10^{-7} , 10^{-7} , and 10^{-8} cc. of broth cultures, respectively.

Experiment 6.—Plain broth was treated with different concentrations of cysteine (0.002 per cent, 0.005 per cent, 0.01 per cent, 0.02 per cent, 0.03 per cent, 0.1 per cent), in order to determine the smallest concentration at which that substance would be active. The tubes were inoculated 12 hours after treatment and incubated under aerobic conditions for 72 hours. The organisms used were those described in the experimental methods.

This experiment shows that cysteine was still active in as high a dilution as 0.005 per cent. With very small inocula (10^{-6} , 10^{-7} , 10^{-8}) growth developed only after 2 to 3 days, this indicating that cysteine was still active at the time.

TABLE VI.

Smallest Inocula (in cc.) with Which Growth Developed in Presence of Different Concentrations of Cysteine.

Organism	Concentration of cysteine						
	0.1	0.03	0.02	0.01	0.005	0.002	0
	<i>per cent</i>	<i>per cent</i>	<i>per cent</i>	<i>per cent</i>	<i>per cent</i>	<i>per cent</i>	
<i>Pneumococcus</i> 1/193/R.....	10 ⁻⁷	10 ⁻⁸	10 ⁻⁸	10 ⁻⁸	10 ⁻⁷	10 ⁻⁴	10 ⁻²
1/200/5.....	10 ⁻⁸	10 ⁻⁷	10 ⁻⁷	10 ⁻⁶	10 ⁻⁶	10 ⁻³	10 ⁻²
D/39/R.....	10 ⁻⁸	10 ⁻⁸	10 ⁻⁸	10 ⁻⁶	10 ⁻⁸	10 ⁻⁵	10 ⁻³
D/39/45.....	10 ⁻⁷	10 ⁻⁷	10 ⁻⁸	10 ⁻⁷	10 ⁻⁸	10 ⁻³	10 ⁻²
M/3/R.....	10 ⁻⁸	10 ⁻⁸	10 ⁻⁸	10 ⁻⁶	10 ⁻⁷	10 ⁻⁴	10 ⁻²
A/66/64.....	10 ⁻⁶	10 ⁻⁸	10 ⁻⁷	10 ⁻⁶	10 ⁻⁶	10 ⁻³	10 ⁻²
<i>S. hæmolyticus</i> S/23/G.....	10 ⁻⁸	10 ⁻⁷	10 ⁻⁷	10 ⁻⁶	10 ⁻⁶	10 ⁻³	10 ⁻²
S/23/76.....	10 ⁻⁷	10 ⁻⁷	10 ⁻⁷	10 ⁻⁷	10 ⁻⁶	10 ⁻⁴	10 ⁻²
<i>Staphylococcus aureus</i>	10 ⁻⁹	10 ⁻⁹	10 ⁻⁸	10 ⁻⁸	10 ⁻⁸	10 ⁻⁷	10 ⁻⁵

TABLE VII.

Effect of Fumaric and Succinic Acids (0.2 Per Cent in Weight) on the Growth of D/39/R.

Medium	Inoculum (in cc.)			
	10 ⁻¹	10 ⁻²	10 ⁻³	10 ⁻⁴
Plain broth.....	+	+	—	—
Plain broth + succinic acid.....	+	+	—	—
Plain broth + fumaric acid.....	+	+	—	—

TABLE VIII.

Smallest Inocula Required for Growth to Develop in Plain Broth and Ascitic Fluid Broth.

Organism	Plain broth	Ascitic fluid broth	
		Heated	Unheated
<i>Pneumococcus</i> (D/39/R).....	10 ⁻¹	10 ⁻²	10 ⁻⁷
<i>S. hæmolyticus</i> (L).....	10 ⁻¹	10 ⁻²	10 ⁻⁶
<i>Staphylococcus aureus</i>	10 ⁻⁴	?	10 ⁻⁹

It is interesting to test the influence of other reducing organic substances. Succinic acid is such a substance, its oxidized form being fumaric acid.

Experiment 7.—The effect of the addition of plain broth (2 weeks old) of fumaric and succinic acids on the aerobic growth of *Pneumococcus* is recorded in Table VII.

As far as the experiment shows, the addition of succinic and fumaric acids does not affect the growth of *Pneumococcus* (D/39/R). The only difference observed was that growth developed in 24 hours in the succinic acid tubes inoculated with 10^{-2} cc., while it took 48 hours in the plain broth and fumaric acid broth with the same inoculum.

Experiment 8.—Ascitic fluid broth (5 per cent ascitic fluid, added to 3 weeks old plain broth) was tested for its value in the initiation of growth of small inocula of facultative anaerobes. In some of the tubes the ascitic fluid had been boiled before addition to the broth. A typical protocol is presented in Table VIII.

These results have been confirmed with many other strains of the same bacterial species. They show that unheated ascitic fluid broth allows the growth of very small inocula of the organisms used. Ascitic fluid seems to be inactivated by boiling.

DISCUSSION.

It is apparent that the growth-promoting properties of plain broth do not remain constant. Fresh broth used within a few hours after its preparation, usually grows *Pneumococcus*, human strains of hemolytic streptococcus, and *Staphylococcus aureus*, even when one or a very few cells are used as inoculum (10^{-7} , 10^{-9} cc. of plain broth cultures). Within 24 hours, the broth has so changed that it can grow the same organisms only if large inocula are used (10^{-2} , 10^{-3} cc.) and after 3 weeks, the broth has become very poor. However, this same broth can be restored to its original value by autoclaving, boiling, or reducing with hydrogen in the presence of palladinized asbestos, as well as by the addition of small amounts of reduced cysteine, but not by the addition of succinic acid. The addition of ascitic fluid and of very small amounts of blood also serves to restore its value.

It is probably fallacious to attempt to explain the results obtained with all these procedures from one single point of view. However, it may be worth while to point out some possible correlations.

Let us first consider the "deterioration" of plain broth on aging. The fact that this "deterioration" can be completely or partially corrected by reducing the medium with hydrogen and with cysteine, or by keeping it under vaseline seal (condition under which the broth is known to develop highly reducing potentials (12)), suggests strongly that this "deterioration" is connected with some oxidation or oxygenation process. The influence of autoclaving and boiling may lead to the same conclusion.

It is hardly probable that the results can be explained by a purely mechanical removal of the oxygen. Studies with indicators of oxidation-reduction potentials have suggested that boiling or autoclaving has more than this purely mechanical effect (12). It has been shown that recently boiled or autoclaved broth maintains the indophenols in a reduced condition for several hours, even under conditions of active aeration. This indicates that the oxidation potential of the broth is lowered either by the reduction of some reversible system of oxidation-reduction, or by the breaking down of a highly oxidized system. A similar conclusion can be reached from a consideration of the results of Experiment 3, in which it is shown that the influence of boiling lasts for over 12 hours—even if the broth is thoroughly aerated.

Blood is an active reducing agent and is also known to possess catalytic properties. Whether the high dilution at which it is still active justifies the assumption that its action is due to a quantitative reduction is problematical. It is possible that the action of blood results in a catalytic breaking down of the peroxides that appear to form in aerated plain broth. In such a case, the inactivity of the iron catalysts used in Experiment 2 would have to be traced to a lack of specific affinity between the iron compounds and the "organic peroxide" of the broth. Finally, it must be remembered that blood introduces formed elements in the medium. It is certain that a bacterial cell adsorbed by, or simply in the vicinity of, a blood corpuscle, finds local conditions markedly different from those prevailing in the rest of the medium. That such conditions are highly reducing is probable.

Little is known of the properties of ascitic fluid. Confirming others

(14), we have found that its reducing capacity is very small, but the factor intensity should be measured by electrometric methods. On the other hand the sample of ascitic fluid used in these experiments gave the peroxidase and catalase tests; it is possible that these catalytic properties were due to the presence of occult blood in the fluid. In such a case, the action of ascitic fluid would be only another example of the activity of small concentrations of blood.

Concerning the reducing intensity of succinic acid, Thunberg (15) reported that equimolecular mixtures of succinic and fumaric acids kept under anaerobic conditions with methylene blue (in the presence of succinodehydrogenase) give only a partial reduction of the dye. This indicates that the system has a very low reduction intensity, much inferior to that of cysteine (which can reduce indigo carmine) and may account for its inactivity. Furthermore, it is probable that questions of specific affinity have to be considered here.

Any problem of growth is of course a problem of reaction between the organism and the environment. So far, our discussion has been concerned only with the environment. Let us now consider the cell as affected by these environmental conditions. We know that the pneumococcus cell is extremely sensitive to changes in the oxygen tension, its thermostable autoxidizable substance becoming alternately oxidized or reduced according to whether the conditions are aerobic or anaerobic, and the thermolabile cellular constituent becoming irreversibly inactivated by slow oxidation. It is to be expected, therefore, that the condition of the cell will be greatly affected by the oxidation potential of the medium. It is also probable that the condition of oxidation or reduction of the cell is not without effect on its ability to multiply.

Our results seem to be best explained by the following hypothesis.

Oxidation processes bear a definite relation to the size of inoculum required to initiate the growth of *Pneumococcus*, *Streptococcus*, and *Staphylococcus*. As we prepare it, plain broth contains reducing autoxidizable substances. In contact with air these substances give rise either to the oxidized form of a reversible oxidation-reduction system, or, by irreversible oxidation, to highly oxidized substances (of the nature of so called "peroxides"). The organisms studied in this work require a medium with a definite range of oxidation-reduction potential for cell multiplication to occur (7, 9, 16). "Oxidized"

broth has a potential outside this range. Such a condition may be corrected in different ways.

(a) The bacterial cell itself is equipped with an active reducing system. When a large inoculum is used, enough reducing substances are introduced (from the bacterial cells and the reduced medium carried with them) to reduce the "oxidized" broth and bring it back to favorable conditions.

(b) Addition of cysteine and blood, and reduction by means of hydrogen, serve the same purpose. Blood may be active also by catalyzing the breaking down of the hypothetical "peroxides."

(c) It is more difficult to see how boiling or autoclaving may bring about the reduction of a reversible system; it is more probable that they cause a breaking down of the oxidized substance.

(d) When incubated under vaseline seal, plain broth develops a high reduction potential (12). It may seem surprising that, under such conditions, the results are not so favorable as those obtained by reducing the broth in some other way. A possible explanation is that the reduction of the "oxidized" substances of the broth under vaseline seal is only a slow process; before they are all reduced by the broth itself, they exert some toxic action on the bacterial cells and a part of the cells of the inoculum are killed (Experiment 5).²

SUMMARY.

The growth of many pathogenic organisms in plain meat infusion broth is possible only when a large inoculum is used.

This requirement is much less strict when the broth cultures are

² It is realized that the oxidation-reduction potential of the broth is only one of many factors to be considered in the problem of initiation of growth. In the course of this work, several batches of plain broth prepared according to the standard method, have been found to be very poor for the growth of *Pneumococcus* and their value could not be enhanced by any of the treatments enumerated above.

It has been observed that samples of broth, several months old, become completely inadequate for the growth of *Pneumococcus* after they have been boiled, but that their value can be restored by addition of cysteine. This effect of boiling on very old broth is not as yet understood.

Recent experiments have shown that cystine could be replaced by equivalent concentrations of thioglycollic acid.

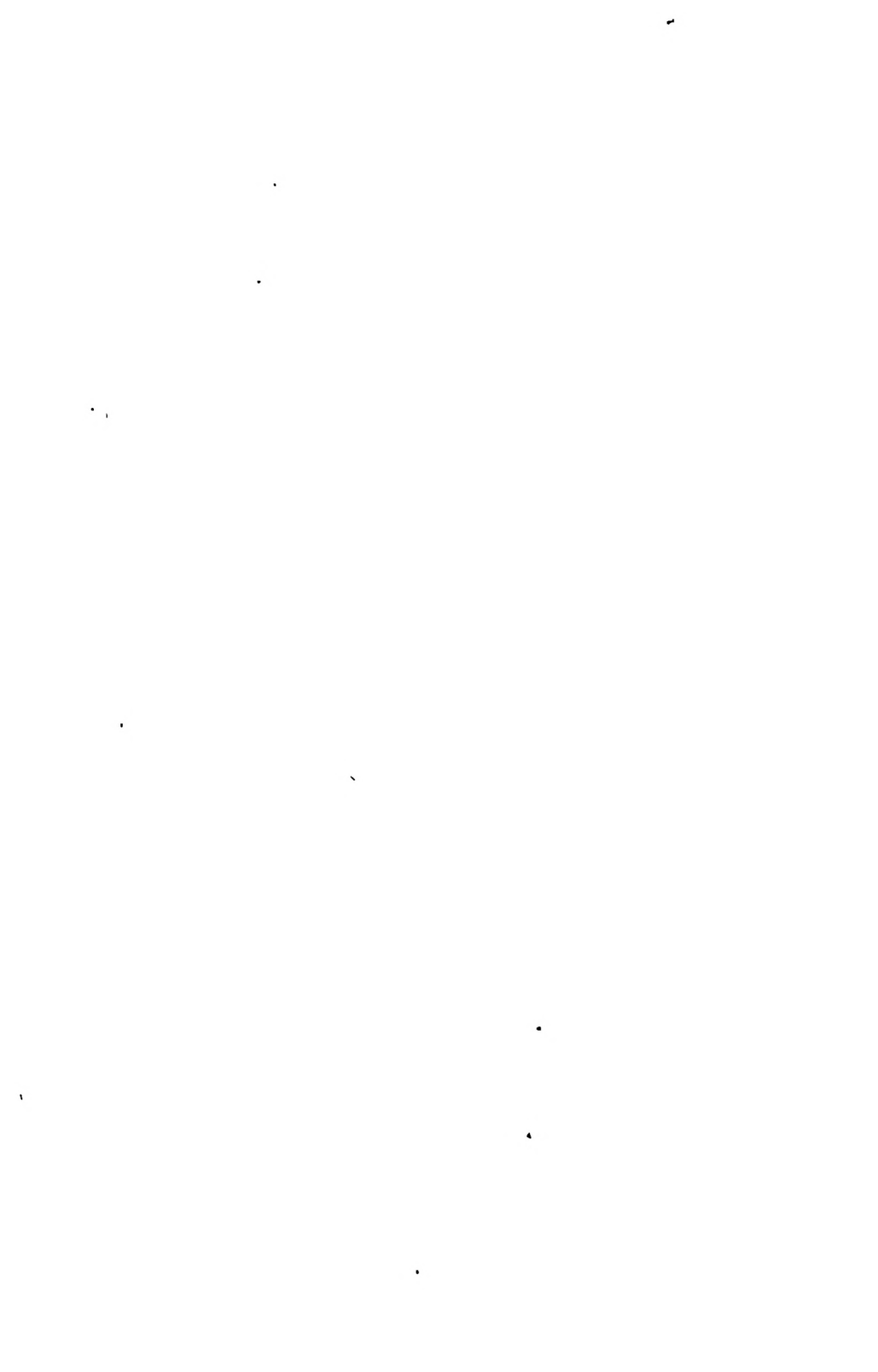
incubated (a) under anaerobic conditions, (b) in fresh media very recently boiled or autoclaved, (c) in fresh media reduced by means of hydrogen, or to which small amounts of cysteine or of blood have been added.

It is suggested that these findings can be accounted for by assuming that the bacterial species under consideration can multiply only in media the oxidation potential of which is below a critical value.

The favorable growth conditions obtained by the procedures enumerated above may be attributed to the establishment of a proper reduction potential in the medium; the same result is obtained by using a large inoculum, owing to the reducing properties of bacterial cells.

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THE RELATION OF THE BACTERIOSTATIC ACTION OF CERTAIN DYES TO OXIDATION-REDUCTION PROCESSES.

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In a preceding paper (1) it has been suggested that the growth of certain facultative anaerobes is inhibited by the existence in the medium of substances with a high oxidation potential. Accurate studies on the critical potentials for different species of bacteria will require the use of media "poised" at definite values. Even then, the results will be significant only if it is demonstrated that there exists a condition of equilibrium between the different parts of the system under consideration.

Unfortunately, we have at our disposal only very few oxidation-reduction systems, the characteristics of which are known. Among these may be mentioned the color indicators of Clark. It seems that these indicators together with a few other systems, such as hemoglobin-methemoglobin, fumaric-succinic acid, hermidin, echinochrome and some bacterial pigments, might be used to "poise" the media at their own characteristic potentials. However, the use of this technique is complicated by the toxicity of some of the dyes for certain bacterial species. It appeared possible that, in some cases, this toxicity might be partly accounted for by the high oxidation potentials of the oxidized dyes. Were this hypothesis justified, it might give us a method of determining the critical potential of the bacterial species studied, by determining which of the dyes are toxic in the oxidized form, and not toxic in the reduced form.

The bactericidal and bacteriostatic actions of certain dyes on different groups of microorganisms has been known for a long time, but the mechanism of this action is still obscure; it is likely that the mechanism varies with each type of dyes and each group of organisms. An

extensive review of the subject has been recently presented by Sartorius (2), but without any special discussion of the fundamental principles involved.

Among the few facts which have been established in this respect may be mentioned the more or less complete parallelism between the bacteriostatic of triphenylmethanes and the Gram reaction (3). On the other hand, azine and azonium dyes, such as phenosafranine and Janus green are known to precipitate proteolytic enzymes (4) and to penetrate the living cell (5). A striking example of the differential toxicity of a dye for two closely related bacterial species has been described by Sherman and Albus (6). They showed that, while the growth of hemolytic streptococci of human and bovine origin is inhibited by the presence in the medium of even low concentrations of methylene blue, the cheese strains thrive in presence of much higher concentrations of the same dye. Later, Avery (7) found that the sample of methylene blue used contained Zn as impurity, and that Zn salts alone, in corresponding concentrations, would also inhibit the human and bovine strains. However, purified methylene blue retained the same property. Some time later, Brown (8) reported, "that streptococci which were inhibited by methylene blue in the presence of oxygen grew well in media containing methylene blue which was reduced."

Burnet (9) studied the growth of certain organisms on acid fuchsin agar plates and found that the growth of "isolated" cells of *Staphylococcus aureus*, diphtheroid bacillus and Friedländer's bacillus was inhibited on such media. (Isolated cells were obtained by spreading a dilute suspension of the culture over the plate.) However, growth developed in the vicinity of colonies of *Staphylococcus aureus* obtained by local heavy inoculation. Burnet concluded from his experiments "that the fuchsin interferes directly with the production or utilization of peroxide by the organism. The presence of products of growth whose primary function is to destroy peroxide, protects isolated organisms against otherwise inhibiting concentrations of the dye. . . . Fuchsin must act by rendering the organism sensitive to traces of peroxide produced in its own metabolism."

Although not dealing directly with the toxicity of dyes for bacterial cultures, the following three studies may be mentioned here.

W. M. Clark and associates (10) have indicated that the halogens lose the greater part of their toxicity when added under conditions which prevent the attainment of oxidation potentials positive to the indophenols. Voegtlin and collaborators (11), working with normal and cancer tissues, found that the "toxic action of dyes depends to some extent on their oxidizing power for reduced glutathione, and also on other features of their molecular structure" and demonstrated by toxicity tests "the existence of a biological antagonism between reduced glutathione and methylene blue." In the course of their studies on the intracellular oxidation-reduction potentials of *Amaba dubia*, Cohen, Chambers and

Reznikoff (12) observed that "the reductants of toxic oxidants were usually non-toxic" and that "the diazines were decidedly toxic."

The ultimate object of the following experiments was to find out whether indicators of oxidation-reduction can be used to determine the critical oxidation potential of the medium above which the growth of *Pneumococcus* and *Streptococcus* cannot take place. The problem which has been most directly considered here is an analysis of the mechanism by which such indicators exert a bacteriostatic action on these species.

TABLE I.
List of Dyes Used.*

Name of oxidant	E_0' (pH = 7.4) (in volts)	rH
2-Chloroindophenol (o-chlorophenol indophenol).....	+0.233	21.8
Indophenol (phenol indophenol).....	+0.228	21.6
2-Methyl indophenol (o-cresol indophenol).....	+0.195	20.5
1-Naphthol-2-sulfonate indophenol.....	+0.123	18.1
Methylene blue.....	+0.011	14.4
Janus green (green \rightarrow pink)..... (approx.)	-0.02	13
K ₄ indigo tetrasulfonate.....	-0.046	12.5
K ₃ indigo trisulfonate.....	-0.081	11.3
K ₂ indigo disulfonate.....	-0.125	9.9
K indigo monosulfonate.....	-0.182	7.5
Janus green (pink \rightleftharpoons colorless)..... (approx.)	-0.26	5
Neutral red..... (approx.)	-0.31	3.7
Phenosafranine..... (approx.)	-0.525	-3.5
Litmus.....	?	?
Malachite green.....	?	?

* The indophenols, the methylene blue, and 3 of the indigoes were obtained from La Motte Chemical Company. Indigo trisulfonate was obtained through the courtesy of Dr. B. Cohen of the Hygiene Laboratory, Washington, D. C. The other indicators were dyes used in the laboratory for staining and cytological work.

EXPERIMENTAL.

Methods.

12 hour plain broth cultures of the following organisms were used in the test
Pneumococcus: smooth virulent: Type I (1/200/4)
 Type II (D/39/43)
 Type III (A/66/69)

Rough avirulent derived from Type I (1/193/R)
 Type II (D/39/R)
 Type III (M/3/R)

Streptococcus haemolyticus: human strains: L, S/43, S/23/Glossy, S/23/Matt
 bovine strains: C/64, C/67
 cheese strains: P, M

Since, in all cases, the results have been the same for all the strains of the same type of organism, only the results dealing with representatives of each type will be given.

The experiments were carried out in meat infusion broth, prepared 1 to 2 weeks before use.

The dyes used may be found in Table I.

The behavior of these dyes in plain broth has been studied in a preceding paper. It may be well to review briefly some of these results.

All the dyes studied function as reversible systems of oxidation-reduction, with the exception of the green \rightarrow pink reduction of Janus green which is irreversible. The rH of all but two of them are known. When added to plain broth in concentrations not exceeding the reduction capacity of the medium, all indicators positive to indigo disulfonate, *i.e.* with an rH higher than 10, are reduced, if the system is kept under vaseline seal. The time of reduction for equimolecular concentrations of the dyes increases progressively as the rH becomes more negative. Although no electrometric measurements are available for malachite green and litmus, the fact that they were not reduced by plain broth suggests that their rH is smaller than that of indigo disulfonate.

Many of the indicators, when added to broth, are rapidly decomposed under aerobic conditions, but remain stable under vaseline seal; the indophenols are especially unstable (particularly methyl indophenol, and phenol indophenol).

The dye solutions were autoclaved, except the solutions of indophenols which were prepared (with aseptic technique) and proved to be sterile.

Aerobic Growth of Pneumococcus and Hemolytic Streptococcus in the Presence of Oxidized Dyes.

Experiment 1.—To a series of test-tubes each containing 5 cc. of broth, sufficient amounts of the different dyes were added to give final concentrations of 0.001 M, 0.003 M, 0.0001 M. The tubes were inoculated with 0.1 to 0.01 cc. of

TABLE II.

Growth of Pneumococcus and Hemolytic Streptococci in the Presence of rH Indicators.

Indicator		Pneumococcus R strain derived from Type II (D/39/R)		Streptococcus hemolyticus, Human strain L		Streptococcus hemolyticus, Cheese strain P	
		Inoculum cc.		Inoculum cc.		Inoculum cc.	
		10 ⁻¹	10 ⁻²	10 ⁻¹	10 ⁻²	10 ⁻¹	10 ⁻²
2-Chloroindophenol	M						
	0.001	—	—	—	—	†*	††
	.0003	—	—	—	—	†	†
Phenol indophenol	.0001	+	—	—	—	†	†
	.001	—	—	—	—	††	††
	.0003	—	—	—	—	†	†
2-Methyl indophenol	.0001	+	—	—	—	†	†
	.001	Erratic results due to breaking down of the dye in the broth				†	†
	.0003					†	†
2-Sulfonate-1-naphthol indophenol	.0001					†	†
	.001	—	—	—	—	†	†
	.0003	—	—	+	—	†	†
Methylene blue	.0001	+	—	+	+	†	†
	.001	—	—	—	—	†	†
	.0003	—	—	—	—	†	†
Janus green	.0001	+	—	—	—	†	†
	.001	—	—	—	—	†	†
	.0003	—	—	—	—	—	—
Indigo tetrasulfonate	.0001	—	—	—	—	—	—
	.001	+	+	+	+	†	†
	.0003	+	+	+	+	†	†
Indigo trisulfonate	.0001	+	+	+	+	†	†
	.001	+	+	+	+	†	†
	.0003	+	+	+	+	†	†
	.0001	+	+	+	+	†	†

In this table + indicates that growth developed in 24 hours; — indicates no growth.

* The dyes are arranged in the order of the electromotive series (see Table I).

† The growth of the cheese strains of *Streptococcus hemolyticus* was delayed in the presence of 2-chloroindophenol and phenol indophenol but finally developed in 36 hours.

TABLE II—*Concluded.*

	M	Pneumococcus R strain derived from Type II (D/39/R)		Streptococcus hemolyticus. Human strain L		Streptococcus hemolyticus. Cheese strain P	
		Inoculum cc.		Inoculum cc.		Inoculum cc.	
		10 ⁻¹	10 ⁻²	10 ⁻¹	10 ⁻²	10 ⁻¹	10 ⁻²
Indigo disulfonate	.001	+	+	+	+	+	+
	.0003	+	+	+	+	+	+
	.0001	+	+	+	+	+	+
Indigo monosulfonate	.001	+	+	+	+	+	+
	.0003	+	+	+	+	+	+
	.0001	+	+	+	+	+	+
Neutral red	.001	—	—	—	—	—	—
	.0003	—	—	—	—	+	—
	.0001	—	—	—	—	+	+
Phenosafranine	.001	—	—	—	—	—	—
	.0003	—	—	—	—	+	+
	.0001	—	—	—	—	+	+
Malachite green	.001	+	+	+	+	+	+
	.003	+	+	+	+	+	+
	.0001	+	+	+	+	+	+
Litmus	?	+	+	+	+	+	+

TABLE III.

Time Required for the Growth of Cheese Strain (P) of Hemolytic Streptococcus to Develop in the Presence of Indophenols.

Dye	Concentration	Inoculum cc.			
		10 ⁻³	10 ⁻⁴	10 ⁻⁵	10 ⁻⁶
	M	hrs.	hrs.	hrs.	hrs.
2-Chloroindophenol.....	0.001	28	48	48	48
Phenol indophenol.....	0.001	28	28	28	28
1-Naphthol-2-sulfonate indophenol.....	0.001	12	18	20	20

the various cultures and growth was recorded after 36 hours incubation at 37°C. Typical protocols are given in Table II.

The results were definite except in the case of the cheese strain of *Streptococcus hæmolyticus*, the growth of which was retarded in the presence of some of the indophenols but finally developed. The question was investigated further by using smaller inocula of this strain (Table III).

Judging from the color of the solution, a good deal of the 2-chloroindophenol was decomposed in 48 hours, so that it may be concluded that the growth of cheese strains of *Streptococcus hæmolyticus* is at least partially inhibited by 2-chloroindophenol, and also perhaps by phenol indophenol.

Summarizing the results of Tables II and III, it appears that the growth of Pneumococcus and of human strains of *Streptococcus hæmolyticus* is inhibited by all the dyes positive to indigo tetrasulfonate (*i.e.* with an rH higher than 12.5), and by neutral red and phenosafranine. The cheese strains of *Streptococcus hæmolyticus* are inhibited only and more or less completely by the most positive indophenols and by Janus green, neutral red and phenosafranine.

Toxic Action of Some of the Oxidized Dyes on Cultures of Streptococcus hæmolyticus.

It is probable that the effect of the addition of dyes to broth is manifold; it is known for instance, that bacteriostatic and bactericidal action of dyes do not always run parallel (3). Before attempting to find whether the bacteriostatic action of the oxidized forms of rH indicators is due in many cases to a "poising" of the medium at a high oxidation potential, it is necessary to eliminate from our tests those indicators which can be shown to exhibit a primary toxicity, not related to oxidation-reduction phenomena. While the object of Experiment 1 was to establish which of the dyes would prevent cell multiplication. Experiment 2 served to establish whether any of these dyes would be toxic for the cells. This was tested by adding dyes to a fully developed culture and studying their effect.

Experiment 2.—12 hour broth cultures of *Streptococcus hæmolyticus* were transferred to test-tubes in amounts of 2 cc. per tube; sufficient amounts of the

dyes were then added to give a final concentration of 0.005 M. After exposure of the cultures to the dyes for different lengths of time, subcultures were made on blood agar plate to determine the viability. The test was not performed with *Pneumococcus* because the cells of this organism autolyze too rapidly.

The results of Experiment 2 are given in Table IV.

These results indicate that, under the conditions of the experiment,

TABLE IV.

Toxic Action of pH Indicators on Streptococcus hæmolyticus.*

Dyes in 0.005 M concentration	<i>Streptococcus hæmolyticus</i>						
	Human strain					Cheese strain	
	Time of exposure (hrs.)					Time of exposure (hrs.)	
	2	4	7	10	28	10	28
2-Chloroindophenol.....	+	+	+	+	+	+	+
Phenol indophenol.....	+	+	+	+	+	+	+
1-Naphthol-2-sulfonate indophenol.....	+	+	+	+	+	+	+
Methylene blue.....	+	+	+	+	+	+	+
Janus green.....	—	—	—	—	—	±	—
Indigo tetrasulfonate.....	+	+	+	+	+	+	+
Indigo trisulfonate.....	+	+	+	+	+	+	+
Indigo disulfonate.....	+	+	+	+	+	+	+
Indigo monosulfonate.....	+	+	+	+	+	+	+
Neutral red.....	+	±	—	—	—	+	—
Phenosafranine.....	+	+	+	+	+	+	+
Litmus.....	+	+	+	+	+	+	+
Malachite green.....	+	+	+	+	+	+	+

* The dyes are arranged in the order of the electromotive series (see Table I).

— indicates no growth on blood agar, ± indicates poor growth on blood agar, + indicates good growth on blood agar.

Janus green, neutral red and phenosafranine exerted a definite toxic action on the cells of *Streptococcus hæmolyticus*. On the contrary, the cells remained alive much longer in the indophenols, methylene blue, the indigos, malachite green and litmus. Further work was therefore limited to this latter group of dyes.

Growth of Pneumococcus and Hemolytic Streptococcus in the Presence of Reduced Indophenols.

If the bacteriostatic action of the oxidized indophenols and methylene blue is due to the fact that they "poise" the medium at a high oxidation potential, the reduced forms of these dyes should be inactive.

Experiment 3.—The bacteriostatic action of the reduced dyes was tested in the apparatus represented in Fig. 1. This apparatus is a slight modification of the one described in a previous article (1) Fig. 2, the tube through which the hydrogen enters the system being shorter in order to avoid bubbling gas through the culture.

The experiment was carried out as follows:

10 cc. portions of recently boiled broth were transferred to each of 36 large tubes; the reduced dyes were added to 12 of them which were filled with the reduction apparatus (Fig. 1); 12 other tubes were treated with the oxidized dyes and the last 6 did not receive any treatment. The dyes (2-chloroindophenol and 1-naphthol-2-sulfonate indophenol) were used in concentrations of 0.0005 μ . All the tubes had been inoculated before treatment. Growth was recorded after 24 hours incubation at 37°C. (Table V).

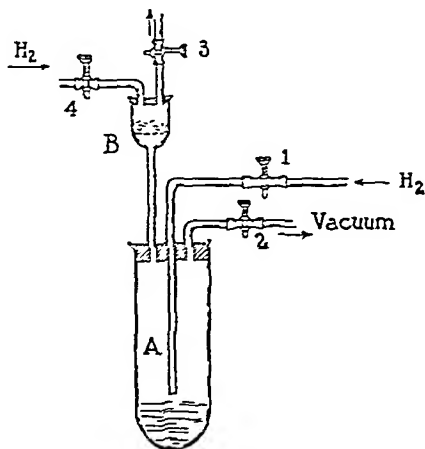


FIG. 1.

This experiment confirms the complete inhibition of growth of *Pneumococcus* and human strains of *Streptococcus hæmolyticus* by the oxidized indophenols and the partial inhibition of the cheese strains by the oxidized 2-chloroindophenol. On the contrary, all the cultures grew well in the presence of the reduced dyes.

The Effect of the Addition of Oxidized Dyes to Plain Broth on the Growth of Pneumococcus and Streptococcus hæmolyticus Incubated under Anaerobic Conditions.

It has been shown (13) that the indophenols and methylene blue are reduced by plain broth when the system is kept under vaseline seal. In consideration of the results of Experiments 3, it appears likely there-

fore that the bacteriostatic action of the dyes would be greatly decreased under vaseline seal.

It is also known that methylene blue is reduced in plain broth when the mixture is kept in anaerobic jars. As was to be expected, the indophenols are also reduced under such conditions.

Experiment 4.—The toxicity of methylene blue and indophenols was tested under vaseline seal and in anaerobic jars. In the course of the experiments, it was found preferable to use smaller concentrations of methylene blue than of indophenols for the two following reasons; (a) methylene blue is reduced only very slowly in high concentration, (b) methylene white being but little soluble precipitates out.

TABLE V.

Influence of the Addition of Oxidized and Reduced Indophenols on the Growth of Pneumococcus and Hemolytic Streptococci.

Dyes in 0.0005 M concentration	Pneumococcus		Streptococcus hemolyticus			
	Inoculum cc.		Human strains		Cheese strains	
			Inoculum cc.		Inoculum cc.	
	10 ⁻¹	10 ⁻²	10 ⁻¹	10 ⁻²	10 ⁻³ *	10 ⁻⁴ *
2-Chloroindophenol, oxidized.....	—	—	—	—	+	—
2-Chloroindophenol, reduced.....	+	+	+	+	+	+
1-Naphthol-2-sulfonate indophenol, oxidized.	—	—	—	—	+	—
1-Naphthol-2-sulfonate indophenol, reduced.	+	+	+	+	+	+
Controls.....	+	+	+	+	+	+

* As already pointed out in Experiment 1, the bacteriostatic action of oxidized indophenols on cheese strains of *Streptococcus hemolyticus* can be recognized only when very small inocula are used.

On the other hand, both the reduced and oxidized forms of the indophenols are very soluble and the oxidized forms are reduced rapidly even in concentrations as high as 0.0005 M; furthermore, these dyes decompose rapidly under aerobic conditions in the presence of broth, and it is necessary to use high concentrations to obtain sharp results.

In this experiment, the indophenols and indigoes were used in 0.0005 M concentrations and methylene blue in 0.00006 M concentrations.

The broths used were 6, 3 and 9 days old, respectively. The cultures were *Pneumococcus* (D/39/R), *Streptococcus hemolyticus*, human strain L and cheese strain P. The size of inocula used and the results are given in Tables VI, VII and VIII. The tubes marked "vaseline" were sealed with a 2 cm. layer of vaseline; the anaerobic jars were incubated for 48 hours before being opened.

The results of Tables VI, VII and VIII constitute a new demonstration of the bacteriostatic action of the oxidized forms of methylene blue and the indophenols on *Pneumococcus* and human strains of

TABLE VI.

Growth of Pneumococcus (D/39/R) in the Presence of Dyes, under Aerobic and Anaerobic Conditions.

Dye	Aerobic					Anaerobic									
						Vaseline seal					Jar				
	Inoculum cc.					Inoculum cc.					Inoculum cc.				
	10 ⁻¹	10 ⁻²	10 ⁻³	10 ⁻⁴	10 ⁻⁵	10 ⁻¹	10 ⁻²	10 ⁻³	10 ⁻⁴	10 ⁻⁵	10 ⁻¹	10 ⁻²	10 ⁻³	10 ⁻⁴	10 ⁻⁵
2-Chloroindophenol 0.0005 M.....	—	—	—	—	—	+	+	+	+	—	+	+	+	—	—
1-Naphthol-2-sulfonate indophenol 0.0005 M.....	—	—	—	—	—	+	+	+	—	—	+	+	—	—	—
Methylene blue 0.00006 M.....	—	—	—	—	—	+	+	—	—	—	+	+	+	+	—
Indigo tetrasulfonate 0.0005 M.....	+	+	—	—	—	+	+	+	+	+	+	+	+	—	—
Plain broth.....	+	+	—	—	—	+	+	+	+	—	+	+	+	—	—

+ indicates that growth developed, — indicates no growth developed.

TABLE VII.

Growth of Human Strains (L) of Streptococcus haemolyticus in the Presence of Dyes, under Aerobic and Anaerobic Conditions.

Dye	Aerobic					Anaerobic									
						Vaseline seal					Jar				
	Inoculum cc.					Inoculum cc.					Inoculum cc.				
	10 ⁻¹	10 ⁻²	10 ⁻³	10 ⁻⁴	10 ⁻⁵	10 ⁻¹	10 ⁻²	10 ⁻³	10 ⁻⁴	10 ⁻⁵	10 ⁻¹	10 ⁻²	10 ⁻³	10 ⁻⁴	10 ⁻⁵
2-Chloroindophenol 0.0005 M.....	—	—	—	—	—	+	+	+	—	—	+	+	+	—	—
1-Naphthol-2-sulfonate indophenol 0.0005 M.....	—	—	—	—	—	+	+	—	—	—	+	—	—	—	—
Methylene blue 0.00006 M.....	—	—	—	—	—	+	+	+	—	—	+	+	+	—	—
Indigo tetrasulfonate 0.0005 M.....	+	+	+	—	—	+	+	+	+	+	+	+	+	+	—
Plain broth.....	+	+	+	—	—	+	+	+	+	+	+	+	+	+	—

+ indicates that growth developed, — indicates no growth developed.

Streptococcus haemolyticus; as expected, the cheese strain of *Streptococcus haemolyticus* was inhibited only by high concentrations of 2-chloroindophenol and when the inoculum was small. Under anaerobic

conditions (vaseline seal or anaerobic jar), methylene blue and the indophenols lost a great part of their bacteriostatic action. In the tubes kept under vaseline seal, it was possible to observe that, in the

TABLE VIII.

Growth of Cheese Strain (P) of Streptococcus hæmolyticus in the Presence of Dyes, under Aerobic and Anaerobic Conditions.

Dye	Aerobic					Anaerobic									
	Inoculum cc.					Vaseline seal					Jar				
						Inoculum cc.					Inoculum cc.				
	10 ⁻¹	10 ⁻²	10 ⁻³	10 ⁻⁴	10 ⁻⁵	10 ⁻¹	10 ⁻²	10 ⁻³	10 ⁻⁴	10 ⁻⁵	10 ⁻¹	10 ⁻²	10 ⁻³	10 ⁻⁴	10 ⁻⁵
2-Chloroindophenol 0.001 M.....	—	—	—	—	—	+	+	+	+	+	+	+	+	+	—
1-Naphthol-2-sulfonate indophenol 0.001 M...	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Plain broth.....	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+

+ indicates that growth developed, — indicates no growth developed.

TABLE IX.

Influence of 0.00002 M Methylene Blue on the Growth of Type II Pneumococcus (D/39/3) in "Boiled" and "Unboiled" Broth under Aerobic and Anaerobic Conditions.

Inoculum cc.	Aerobic				Anaerobic Jar			
	"Unboiled" broth		"Boiled" broth		"Unboiled" broth		"Boiled" broth	
	Nomethyl- ene blue	Methyl- ene blue	Nomethyl- ene blue	Methyl- ene blue	Nomethyl- ene blue	Methyl- ene blue	Nomethyl- ene blue	Methyl- ene blue
10 ⁻¹	+	+	+	+	+	+	+	+
10 ⁻²	+	—	+	+	+	+	+	+
10 ⁻³	—	—	+	—	—	+	+	+
10 ⁻⁴	—	—	+	—	—	+	+	+
10 ⁻⁵	—	—	+	—	—	+	+	+
10 ⁻⁶	—	—	+	—	—	—	+	+

+ indicates that growth developed, — indicates no growth developed.

presence of these dyes, growth did not develop as long as the dye was not reduced. On the contrary, growth developed in the presence of the oxidized form of indigo tetrasulfonate.

The unexpected observation has been made that the initiation of growth of *Pneumococcus* is facilitated in the anaerobic jar when traces of methylene blue are added to the broth. The most characteristic example of such an effect is given in Table IX.

Experiment 5.—One half of the broth used in this experiment had been boiled immediately before inoculation. The "boiled" and "unboiled" samples were divided again into two series; one of them was inoculated as such, the other one only after addition of 0.00002 M methylene blue. The inoculated tubes were incubated either under aerobic conditions, or in an anaerobic jar. The observations were made after 36 hours incubation (Table IX).

Similar results have been obtained with 6 different strains of *Pneumococcus*. They confirm that methylene blue is not toxic, when reduced in the broth. In the anaerobic jar, growth developed only with 10^{-1} and 10^{-2} cc. inoculum in the plain "unboiled" broth, while it took place with as little as 10^{-5} cc. inoculum in the presence of 0.00002 M methylene blue. However, even such a minute concentration of the dye is bactericidal in the oxidized form, as appears from the results obtained under aerobic conditions. (This is especially striking in the case of "boiled" broth.) The growth of very small inocula in the presence of traces of methylene blue in the anaerobic jar must be due therefore to an indirect effect. It can be explained by assuming that methylene blue, being such an actively reversible system, does accelerate the reduction of the broth itself.

Comparative Growth of Human and Cheese Strains of Streptococcus hæmolyticus in Plain Broth.

It has been established that the growth of human strains of *Streptococcus hæmolyticus* is completely checked by the presence in the medium of the oxidized forms of methylene blue and the indophenols, while the growth of the cheese strains of *Streptococcus hæmolyticus* is inhibited only by the 2-chloroindophenol, the most positive of the indicators tested. On the other hand, it has been suggested elsewhere (1) that the growth of small inocula of human strains of *Streptococcus hæmolyticus* may be inhibited by the presence of oxidized substances in plain broth. It was interesting to compare the growth of small inocula of human and cheese strains of *Streptococcus hæmolyticus* in this medium.

Experiment 6.—Varying amounts of 12 hour cultures of human (L) and cheese (P) strains of *Streptococcus hæmolyticus* were seeded into test-tubes containing 5 cc. of plain broth (1 week old). Growth was recorded after 24 hours (Table X).

The results of Table X indicate that, while a fairly large inoculum is necessary to insure the growth of human strains of *Streptococcus hæmolyticus* in plain broth, the cheese strains develop in the same medium following inoculation of a very few cells only.

DISCUSSION.

Janus green, neutral red and phenosafranine form a special class among the indicators of oxidation-reduction potential used in this work.

TABLE X.

Growth of Human (L) and Cheese (P) Strains of Streptococcus hæmolyticus in Plain Broth.

<i>Streptococcus hæmolyticus</i>	Inoculum cc.						
	10 ⁻¹	10 ⁻²	10 ⁻³	10 ⁻⁴	10 ⁻⁵	10 ⁻⁶	10 ⁻⁷
Cheese strain P.....	+	+	+	+	+	+	+
Human strain L.....	+	+	—	—	—	—	—

+ indicates that growth developed, — indicates no growth developed.

They rapidly kill cultures of hemolytic streptococci to which they are added in sufficiently high concentrations. These cultures remain alive for a longer time when put in contact with the same concentrations of the other indicators. This toxic action of Janus green, neutral red and phenosafranine is not related to phenomena of oxidation-reduction. The discussion will therefore be limited to the other indicators.

The results concerning the bacteriostatic action of the indophenols, methylene blue, the indigoes, malachite green and litmus on *Pneumococcus* and *Streptococcus hæmolyticus* are summarized in Table XI.

In Table XI, the dyes are arranged in the order of their oxidation-reduction potentials (electromotive series). It is apparent that *Pneumococcus* and human strains of *Streptococcus hæmolyticus* are inhibited only by the indophenols and methylene blue; *i.e.* by the dyes having an rH higher than 12.5. The cheese strains of *Streptococcus*

hæmolyticus are inhibited by 2-chloroindophenol and perhaps phenol indophenol, but not by the others, indicating that their "bacteriostatic potential" is above rH 21.

The bacteriostatic action of the indophenols and methylene blue is completely or partly corrected when (a) they are added to the medium in the reduced form, (b) the mixture broth-dye is incubated under anaerobic conditions (the concentration of dyes being low enough to allow their reduction in a reasonable time).

TABLE XI.

Growth of Pneumococcus and Hemolytic Streptococci in the Presence of Different rH Indicators Used in the Oxidized Form under Aerobic Conditions.

Dye*	Pneumococcus	Streptococcus hæmolyticus	
		Human strain	Cheese strain
2-Chloroindophenol.....	—	—	+ —
Phenolindophenol.....	—	—	+ — (?)
1-Naphthol-2-sulfonate indophenol.....	—	—	+
Methylene blue.....	—	—	+
Indigo tetrasulfonate.....	+	+	+
Indigo trisulfonate.....	+	+	+
Indigo disulfonate.....	+	+	+
Indigo monosulfonate.....	+	+	+
Malachite green.....	+	+	+
Litmus.....	+	+	+

* The dyes are arranged in the order of the electromotive series (see Table I).

— indicates complete inhibition of growth, + — indicates partial inhibition of growth, + indicates normal growth.

It has already been suggested that, before an organism can multiply, the medium in which it is present must reach a critical reduction potential. It is attractive to suppose that, for *Pneumococcus* and human strains of hemolytic streptococcus, the critical potential is between indigo tetrasulfonate (rH = 12.5) and methylene blue (rH = 14.4) while it is somewhere below the chloroindophenol for the cheese strains of *Streptococcus hæmolyticus*. The corollary of such a view is that all the dyes or other substances with an oxidation potential higher than that of indigo tetrasulfonate would be bacteriostatic for *Pneumococcus* and human strains of hemolytic streptococcus, while

only the ones positive to phenol indophenol would have such an action on cheese strains of hemolytic streptococcus (provided that the dye is not primarily toxic and that conditions of equilibrium exist between the dye and the culture).

In terms of this hypothesis, the large inocula required for obtaining a growth of Pneumococcus and of human strains of *Streptococcus haemolyticus* in plain broth, serve the purpose of reducing the broth to a potential ($rH = 13$) corresponding to reduced methylene blue. The cheese strains of *Streptococcus haemolyticus* on the contrary grow with such a small inoculum because the potential of aerated broth is within the range in which these organisms can multiply. In fact, the oxidation potential of the broth is certainly not much higher than that of oxidized 2-chloroindophenol, since the dye remains reduced for a long time in broth that has been recently boiled, and aerated afterwards. In fact, 2-chloroindophenol is the only dye tested which exerts a definite bacteriostatic action, and even then, this action is only partial, indicating that we are just on the border of the critical potential.

The fact that the dyes are not toxic in the reduced form is of course in agreement with the hypothesis.

Let us now consider a few of the objections that may be made to this point of view. When the dyes are added in the reduced form, they remain reduced in the medium only if the broth is at an oxidation potential below, or equal to, that of the reduced dye. On the other hand, if oxidized indophenols or methylene blue are added to reduced broth, and the system kept under aerobic conditions to prevent reduction of the dye, it is likely that the broth will oxidize rapidly. Consequently, when we are dealing with oxidized or reduced dye, we are dealing at the same time with oxidized or reduced broth. As we know that the condition of the broth is of the greatest importance for initiation of growth (1), it is hard to know whether the dye is no longer toxic because present in the reduced form or only because the reduced broth is a more favorable medium. To this objection one can answer by pointing out that according to the hypothesis, both oxidized broth and the oxidized indicators under consideration are bacteriostatic, *not as broth or dye*, but by virtue of their *oxidizing intensity*. In both cases, fundamentally, the mechanism of action is the same, and a solution of the problem will be possible only when we have at our disposal media which do not function themselves as active systems of oxidation reduction.

Another weak point is that toxicity stops exactly with the indigoes. Is it not possible that toxicity is related, not to the oxidation-reduction characteristics of the dyes, but to their structure? A convincing proof would be to find an organism inhibited only by some of the indigoes, and not by the ones with a more negative rH. An interesting evidence of this sort is supplied by the fact that cheese strains of *Streptococcus hæmolyticus* are inhibited only by the most positive of the indophenols tested. In that respect, it would be important to test the action of other indophenols.

As they stand, our experiments do not supply any fact to contradict the hypothesis that methylene blue and oxidized indophenols are bacteriostatic because they "poise" the medium at an oxidation potential outside the range in which *Pneumococcus* and human and bovine strains of hemolytic streptococcus can grow. The problem of the mechanism whereby such a medium, poised at a high oxidation potential, becomes bacteriostatic for certain species, can be considered from at least two points of view. On the one hand, we may suppose that the presence of oxidized dyes in the medium results in such a change in the condition of some of the constituents of the broth, that they are no longer available for growth. On the other hand, the action of the dye may be not on the broth, but on the bacterial cells themselves. We know for instance, that the pneumococcus cell contains a dual system of oxidation-reduction consisting of a thermolabile cellular constituent, and thermostable autoxidizable substances which can be removed by washing (14). By means of this system, the *Pneumococcus* cell is able to carry on a series of oxidations and reductions, the thermostable components becoming reversibly reduced or oxidized in the process, while, under aerobic conditions, the cellular component is inactivated by oxidation. It may be considered that the oxidized dyes bring the autoxidizable substances, or the thermolabile cellular constituent, to an oxidation potential where they are no longer adjusted to the metabolic activities of the cell.

Whatever its explanation may be, the fact remains that the indicators studied are much less toxic under anaerobic than under aerobic conditions. The significance of this observation for the use of dyes in therapeutics is evident since their action "*in vitro*," under aerobic conditions, is little indication of what it will be in the presence of living tissues which are known to be active reducing agents. It is to be

expected that some dyes will be very effective when used on superficial wounds, but not when injected.

Undoubtedly, the mode of action of dyes is manifold, but the observations presented above may help in the analysis of this action, by pointing to one of the factors to be controlled.

SUMMARY.

Oxidized indophenols and methylene blue are bacteriostatic for *Pneumococcus* and hemolytic streptococci of human and bovine origin, while the indigoes, malachite green and litmus are not toxic.

2-Chloroindophenol, the most positive of the indicators of oxidation-reduction potentials used, is also the only one to have a bacteriostatic action on cheese strains of *Streptococcus hæmolyticus*.

Methylene blue and the indophenols are no longer bacteriostatic when present in a reduced form in a medium capable of maintaining them in such a condition.

A comparison of these results with the growth in plain broth of the organisms studied suggests that the "inhibiting" dyes "poise" the medium at an oxidation potential outside the range in which the inhibited organisms can grow.

The validity of this hypothesis is discussed.

The significance of these observations for the use of dyes in therapeutics is considered.

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STUDIES ON BACILLUS TYPHOSUS TOXIC SUBSTANCES.

II. THE EFFECT OF SERA UPON THE FACTORS DETERMINING LOCAL SKIN REACTIVITY TO FILTRATES OF BACILLUS TYPHOSUS CULTURES.*

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In the previous communication (1) a local skin reactivity to *B. typhosus* culture filtrates was described. The reactivity was induced by injection of the filtrate into the skin. If 24 hours later an intravenous injection of the same filtrate was given to the rabbit, there appeared an extremely severe hemorrhagic necrosis at the site of the previous injection. The mechanism is not fully understood as yet since no complete experimental comparison has been made between this phenomenon and the manifestations of bacterial allergy. There were found, however, certain features which, *considered together*, distinguish this phenomenon from the known phenomena of bacterial hypersusceptibility and from the Arthus phenomenon. These features are: the local reactivity; the short incubation period necessary for local preparation of the skin; the short duration of the state of reactivity; the ability to induce the reactivity by a single injection; and the necessity to make the second injection of the toxic agent by the intravenous route.

The present paper deals with the relation of the specific antisera to the skin-preparatory factors of the phenomenon above described. There was a double purpose in these studies, first, to elucidate the mechanism of the phenomenon, and secondly, to determine whether specific sera neutralized the preparatory factors, and if so, whether this could be advantageously applied to titration of the neutralizing

* A preliminary report of this work was presented before the Society of American Bacteriologists, Richmond, Virginia, December, 1928.

properties of the sera. In pursuance of this plan the effect of homologous, normal and heterologous sera was studied.

Methods.

Preparation of Immune Antisera.—Most sera were prepared by the injection of toxic culture filtrates. Since a considerable number of the rabbits died from intensive immunization with these untreated filtrates, some animals were injected at the beginning of the immunization with autoclaved filtrates and later with filtrates heated in the Arnold steam sterilizer as also with filtrates heated at 60° for 1–2 hours, and finally with potent toxic filtrates. Some animals received injections of whole bacteria, first heated to 60° for 2 hours, then live bacteria.

Most of the injections were made intravenously, but in some instances the subcutaneous route was employed. They were usually made every week for 3 or 4 successive days for a period of 6–7 weeks. The final bleedings were made about 10 days after the last injection or earlier if the animals appeared sick.

Heterologous sera were similarly prepared. *B. typhosus*, Para A and B, and Shiga bacillus sera employed in some experiments, were prepared in the New York Board of Health Laboratories by immunization of horses. Other animals employed in this work for immunization purposes were goats and rabbits.

Technique of the Neutralization Experiments.—The toxic filtrates were mixed with sera in the desired proportions and the mixtures were incubated in the water bath at 37° for 1 hour. Precipitations were always recorded. Immediately before injection the tubes were shaken and the fluid together with the precipitate was used for injections. The skin of the abdomen of rabbits was prepared, as described in the first paper of this series (1), and the mixture injected into various areas, usually the lower right and upper and lower left areas of the shaven skin of the abdomen. The upper right area of the skin of the abdomen of each rabbit was injected with the toxic filtrate alone, previously diluted with 0.9 per cent sodium chloride solution and kept in the water bath at 37° for 1 hour. Sometimes the order of the injections was changed. - 24 hours afterwards, the *B. typhosus* culture filtrate was injected intravenously and readings of the reactions were made 4–5 hours after the intravenous injections. It may be stated here that the specific sera had no obvious neutralizing effect upon the erythema frequently produced by the skin injections alone. Data on this point are, therefore, omitted from the experimental part of this paper.

EXPERIMENTAL.

A. Effect of Immune Sera upon the Factors Determining Local Skin Reactivity.

About 16 per cent of rabbits in this series gave no reaction and are accordingly omitted from Table I which represents a summary of the

neutralization experiments performed according to the technique described above. Reports on the rabbits that died earlier than 3 hours after the intravenous injection are also omitted for purposes of clarity.

The doses of *B. typhosus* culture filtrates used for the skin and intravenous injections were kept approximately constant.¹ Since some of the rabbits showed from time to time positive reactions elicited by neutralized mixtures, it was necessary to employ several rabbits for each serum tested. Moreover, since high concentrations of serum

TABLE 1.

Neutralization of B. typhosus Skin-Preparatory Factors by Immune Sera.

Sera titrated	Total number of rabbits	Number showing neutralization with the sera; and dilutions of the latter					Number showing no neutralization
		1:2000	1:200	1:100	1:20	1:2	
No. 446	13	6	4	—	1	—	2
No. 90	10	3	3	—	—	—	4
No. 59	10	2	4	—	1	1	2
Typhoid horse serum	9	—	6	—	1	—	2
No. 78	10	—	4	2	1	—	3
No. 165. Goat typhoid serum	10	—	—	—	3	3	4

Sera 446, 90, 59, 78 and Goat 165 were prepared by immunization with *B. typhosus* culture filtrates. Typhoid horse serum obtained from the New York Board of Health was prepared by immunization with dead and live *B. typhosus*.

In all of these rabbits there was a positive reaction in the control areas.

occasionally failed to neutralize (prozone?), whereas higher dilutions of the same serum and in the same rabbit gave complete neutralization the use of several dilutions of serum was necessitated.

The results can be summarized as follows:

There are two types of response to the injection of mixtures of specific sera with the skin-preparatory factors, as demonstrated by

¹ The *B. typhosus* culture filtrate (see page 600 for preparation) was diluted 1:2 for skin injection. 0.25 cc. of filtrate in this dilution represents two minimal skin-preparatory doses, each of sufficient strength to prepare for a severe hemorrhagic necrosis. The intravenous dose was 2 cc. per kilo of body weight in most instances.

intravenous injections 24 hours later, namely, neutralization of the reaction and non-neutralization. Out of a total of 316 tests, there were 135 neutralizations by sera in various dilutions and 181 failures to neutralize. This brings out the fact that neutralization of *B. typhosus* skin-preparatory factors by specific sera can be induced at will in a large proportion of experimental rabbits (about 42.5 percent).

Varying the site of the injection was without influence on the result.

After establishing definitely the fact that neutralization of skin-preparatory factors by specific sera is possible it appeared desirable to analyze the results further in order to determine whether the phenomenon could be advantageously used for titrating the neutralizing power of specific sera. The data obtained with a given immune serum in a large number of rabbits were examined more closely. As is seen from Table I, in six rabbits receiving *B. typhosus* horse serum complete neutralization was obtained in dilution of 1:200, in one rabbit in dilution 1:20, while in two rabbits there was no neutralization. When analysis of the sort is practiced in connection with the other sera it becomes evident that there is a definite variation in the response of different rabbits to the skin-preparatory effect of serum-filtrate mixtures and that the rabbits showing these varying responses can be classified under three groups, namely, those which yield completely neutralized reactions with the highest dilutions (HN) of serum,² those which give rise to completely neutralized reactions only with sera in lower dilutions (LN) and those which show non-neutralized reactions (NN).

Possibly some non-neutralizable fraction of the culture filtrate to which certain rabbits are particularly susceptible may account for the occurrence of non-neutralization. Furthermore, it appears from Table I that the potency of a given serum bears a mathematical ratio to the reactions obtained in the three groups of rabbits. Thus if complete neutralization is obtained, for example, in dilution 1:2000 in HN rabbits, namely, in those showing the highest titer of neutralization, the titer in LN rabbits is likely to be 1:200. In the cases in

² In a preliminary report (3) recently published, HN, LN and NN were called CN, PN and NN respectively. It is now felt that the nomenclature here suggested is more satisfactory.

which the HN is 1:200 the LN titer is only likely to be 1:20. Moreover, the relative numbers of HN, LN and NN rabbits also varies.

As control to the foregoing the effects of a number of normal sera were studied:

B. Effect of Normal Sera upon the Factors Determining Local Skin Reactivity.

Normal sera from horses, goats and rabbits failed to neutralize the skin-preparatory factors in a large percentage of rabbits. It is of considerable interest, however, that some of the normal animals whose

TABLE II.

Sera titrated	Total number of rabbits	Number showing complete neutralization; and the dilutions of the sera			Number showing no neutralization
		1:200	1:20	1:2	
Normal horse serum	17	—	—	—	17
Normal Rabbit Serum 353	8	—	—	—	8
Normal Rabbit Serum 355	8	—	1	2	5
Normal Rabbit Serum 336	3	—	—	—	3
Normal Goat Serum 166	6	—	—	—	6

In addition there were several other normal sera not included in this table which were tested in 2-3 rabbits, with no neutralization obtained.

In all of these rabbits there was a positive reaction in the control area.

serum failed to neutralize the skin-preparatory factors were subsequently injected with *B. typhosus* culture filtrate and responded with the production of neutralizing sera of high titer.

Table II shows, furthermore, that one normal serum was able to neutralize the skin-preparatory factors in low dilutions. It is seen, however, that this normal serum contained a fairly high titer of agglutinins against *B. typhosus* (Table IV). Furthermore, those sera which failed to neutralize the skin-preparatory factors contained no agglutinins or precipitins. Again, some sera which contained agglutinins failed to neutralize skin-preparatory factors.

TABLE III.

The Effect of Heterologous Sera upon the B. typhosus Skin-Preparatory Factors.

Sera titrated	Total number of rabbits	Number showing complete neutralization; and the dilutions of the sera			Number showing no neutralization
		1:200	1:20	1:2	
<i>Paratyphosus</i> A rabbit serum	8	2	1	2	3
<i>Paratyphosus</i> B rabbit serum	8	3	1	—	4
<i>B. coli</i> rabbit serum	10	—	—	—	10
Shiga bacillus horse serum	10	—	—	—	10
Flexner bacillus rabbit serum	10	—	—	—	10
Mt. Desert bacillus rabbit serum	8	—	—	—	8
<i>B. avicida</i> rabbit serum	9	—	—	—	9
Scarlet fever antitoxin	8	—	—	—	8

In all of these rabbits there was a positive reaction in the control area.

TABLE IV.

Agglutination of B. typhosus by Various Sera.

Sera tested	Degree of agglutination with various dilutions of sera and a constant amount of <i>B. typhosus</i> suspension										
	1:4	1:8	1:16	1:32	1:64	1:128	1:256	1:512	1:1024	1:2028	1:4056
Serum 446.....	4+	4+	4+	4+	4+	4+	4+	4+	4+	4+	—
Serum 59.....	4+	4+	4+	4+	4+	4+	4+	4+	4+	NT	NT
Serum 78.....	4+	4+	4+	4+	4+	4+	4+	3+	3+	NT	NT
Serum 90.....	4+	4+	4+	4+	4+	4+	4+	4+	4+	4+	—
Goat typhoid serum.....	4+	4+	4+	4+	4+	3+	2+	1+	—	—	—
<i>Paratyphosus</i> A rabbit serum.....	4+	4+	4+	4+	4+	3+	2+	1+	—	—	—
<i>Paratyphosus</i> B rabbit serum.....	4+	4+	4+	4+	4+	4+	2+	—	—	—	—
<i>B. coli</i> rabbit serum.....	4+	4+	4+	4+	2+	—	—	—	—	—	—
Shiga horse serum.....	—	—	—	—	—	—	—	—	—	—	—
Flexner rabbit serum.....	4+	4+	4+	3+	2+	—	—	—	—	—	—
Mt. Desert rabbit serum.....	4+	4+	4+	2+	1+	—	—	—	—	—	—
<i>Avicida</i> rabbit serum.....	4+	4+	4+	4+	3+	—	—	—	—	—	—
NRS 336.....	4+	4+	4+	4+	4+	4+	4+	4+	4+	NT	NT
NGS 166.....	3+	4+	4+	4+	4+	4+	4+	4+	NT	NT	NT
NRS 353.....	4+	4+	4+	4+	2+	—	—	—	—	—	—
NRS 355.....	—	—	4+	4+	4+	4+	3+	2+	—	—	—
Normal horse serum..	4+	4+	4+	4+	—	—	—	—	—	—	—

—, negative.

Degree of agglutination recorded in pluses.

All these facts indicate that normal sera contain no neutralizing antibodies against the skin-preparatory factors unless they also contain agglutinins against *B. typhosus*. Conversely, however, the presence of agglutinins does not necessarily indicate that the serum possesses neutralizing antibodies against the skin-preparatory factors.

TABLE V.
Precipitation of Various Sera by B. typhosus Culture Filtrate.

Sera tested	Degree of precipitation with various dilutions of sera and a constant amount of T _L filtrate (undiluted)								
	1:2	1:4	1:8	1:16	1:32	1:64	1:128	1:256	1:512
Typhoid horse serum.....	4+	4+	3+	3+	2+	1+	—	—	—
Serum 446.....	4+	4+	4+	3+	3+	2+	2+	1+	—
Serum 59.....	2+	2+	1+	1+	—	—	—	—	—
Serum 78.....	4+	4+	4+	3+	2+	—	—	—	—
Serum 90.....	4+	4+	4+	2+	1+	—	—	—	—
Goat typhoid 165 serum.....	2+	2+	1+	—	—	—	—	—	—
<i>Paratyphosus</i> A rabbit serum.....	4+	4+	4+	4+	4+	3+	2+	1+	—
<i>Paratyphosus</i> B rabbit serum.....	4+	4+	2+	1+	—	—	—	—	—
<i>Coli</i> rabbit serum.....	—	—	—	—	—	—	—	—	—
Shiga horse serum.....	—	—	1+	1+	±	—	—	—	—
Flexner rabbit serum.....	3+	2+	1+	—	—	—	—	—	—
Mt. Desert rabbit serum.....	±	±	—	—	—	—	—	—	—
<i>Arvicola</i> rabbit serum.....	—	—	—	—	—	—	—	—	—
NRS 336.....	—	—	—	—	—	—	—	—	—
Normal Goat Serum 166.....	2+	1+	1+	—	—	—	—	—	—
NRS 353.....	—	—	—	—	—	—	—	—	—
NRS 355.....	—	—	—	—	—	—	—	—	—
Normal horse serum.....	—	—	—	—	—	—	—	—	—

C. Effect of Heterologous Sera upon the Factors Determining Local Skin Reactivity.

A number of heterologous sera were also employed for neutralization experiments (Tables III and IV). Only sera strongly agglutinating their homologous strains were used in this part of the work.

Scarlet fever, erysipelas, *B. coli*, Shiga bacillus, Flexner bacillus, Mt. Desert bacillus and *B. avicida* sera produce no neutralization of the *B. typhosus* skin-preparatory factors (Table III), in spite of the fact that some of the sera contained agglutinins against *B. typhosus* (Table IV). The closely allied sera (Para A and B) were able to

neutralize the *B. typhosus* skin-preparatory factors in various proportions and, as would be expected, agglutinated *B. typhosus* in a fairly high titer. The rabbits employed in these experiments can also be divided into three groups, namely, those showing neutralization in highest dilutions (HN), those showing complete neutralization only in lower dilutions (LN) and those showing no neutralization (NN).

The question arises whether the *B. typhosus* skin-preparatory factors are the same as those of Para A and B, or are only closely related. This problem cannot as yet be decided.

The following scheme is tentatively proposed for the preparation of toxic filtrate and the titration of serum:

200 cc. of tryptic digest broth (2) of initial pH 7.8 are poured to a "2000 cc." Erlenmeyer flask in order to obtain a large surface area. The entire growth of one 24 hour old agar slant culture of *B. typhosus* (T_L)³ is suspended in about 10 cc. of salt solution and the emulsion is added to the contents of the Erlenmeyer flask. The period of incubation is 6 days. The culture is then filtered through paper and cotton and, finally, through a Berkefeld V candle. The filtrate is tested for sterility, stored in the refrigerator and used for a period of 2-3 weeks following its preparation. No preservative is added.

For neutralization experiments 0.25 cc. of a mixture of equal parts of filtrate and saline, or of serum⁴ already diluted to the desired degree, is injected into various areas of the skin of the rabbit's abdomen, by means of the technique described on page 594. The interval between the skin and intravenous injections should be exactly 24 hours. This is very important (1) to obtain uniform results. Both the size and intensity of the reactions should be recorded. A variation in the size of the reaction within small limits can be disregarded. The intensity of the reaction is an important and reliable indication of the degree of neutralization. It may be recorded in pluses. Occasionally, discolored punctiform reactions are obtained. They should be protocolled as such. There should be obtained not less than ten rabbits showing control positive reactions with the mixture of the filtrate with salt solution.

The following schematic formula has been found useful in recording the varying factors obtained in a typical serum titration: HN₆2000, LN₄200, LN₁20, NN₃.

³ The strain of *B. typhosus* employed in this laboratory is designated T_L.

⁴ The majority of the sera were prepared by injecting filtrates into rabbits and goats. Some sera were obtained by injecting dead and live microorganisms, for example typhoid horse serum and some heterologous sera employed in experiments described before. It seems that the presence of intact bacterial cells in the antigens injected is not essential for the production of the above described antibodies, since sera of even higher titer were obtained by means of filtrates alone. Additional experiments are necessary.

This indicates that 6 rabbits showed complete neutralization with serum diluted 1:2000, 4 rabbits showed complete neutralization with serum diluted 1:200, 1 rabbit with serum diluted 1:20 and 2 rabbits showed no neutralization. For practical purposes the highest dilution of the serum which gives complete neutralization of the *B. typhosus* skin-preparatory factors (HN titer) should be taken as the actual titer of the serum. In the example cited, 1:2000 would be considered the titer.

SUMMARY AND CONCLUSIONS.

It is shown in this paper that homologous immune sera are able to neutralize the *B. typhosus* skin-preparatory factors. The neutralization experiments were performed on a large number of rabbits, at least ten rabbits which showed positive control reactions being used for the titration of each serum. The rabbits into which the mixtures of *B. typhosus* culture filtrates with immune sera were injected can be divided into the following categories: those showing complete neutralization in highest dilutions (HN), those showing complete neutralization only in lower dilutions (LN) and those showing no neutralization (NN). The results indicate that the potency of a given serum as measured by the method outlined above has a direct relation to the reactions obtained in these groups of rabbits. For practical purposes the highest dilution of the serum which gives complete neutralization of the *B. typhosus* skin-preparatory factors (HN titer) may be taken as the actual titer of the serum as expressed in terms of their neutralization.

The occurrence of a phenomenon suggestive of the prozone reaction is demonstrated. It also appears that the filtrates possess an antigenicity equal to that of dead and live bacteria.

The studies on normal sera bring out the fact that normal sera fail to neutralize the *B. typhosus* skin-preparatory factors unless agglutinins can be demonstrated for *B. typhosus*. No normal sera have thus far been obtained which neutralized the skin-preparatory factors yet contained no *B. typhosus* agglutinins, but there were sera which contained these agglutinins but failed to neutralize the skin-preparatory factors. Some of the normal animals whose sera failed to neutralize the skin-preparatory factors were subsequently injected with *B. typhosus* culture filtrate and responded with neutralizing sera of high titer.

Several heterologous sera were also investigated namely, scarlet fever, erysipelas, Shiga bacillus, Flexner bacillus, Mt. Desert bacillus, *B. coli* and *B. avicida*. These did not neutralize the *B. typhosus* skin-preparatory factors.

Paratyphosus A and B sera on the other hand produced neutralization in various proportions. And the rabbits into which the serum-filtrate mixtures were injected could also be divided according to the results obtained into the same three groups as those with *B. typhosus* sera. It is not known yet whether this neutralization is a group reaction or whether the skin-preparatory factors are identical with those of *B. typhosus*.

It would appear from these studies that a method is available for the quantitative titration of substances in the serum which neutralize the skin-preparatory factors of local skin reactivity to *B. typhosus* culture filtrates. It should be emphasized that it is possible to control the individual susceptibility of rabbits to this phenomenon. The method should permit of considerable accuracy in the quantitative titration of the neutralizing properties of a serum when a standardized procedure is developed.

Experiments are under way to determine whether the method can be applied to the preparation of therapeutic sera. Work is also in progress to determine the effect of specific antisera upon *B. typhosus* skin-reacting factors introduced by the intravenous route.

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BLOOD CHLORIDES IN CONDITIONS ASSOCIATED WITH PNEUMONIA.

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INTRODUCTION.

The changes which occur in the chloride concentration of the blood and in chloride elimination in the urine in pneumonia have been much studied. No attempt will be made here to review the literature completely. References may be found in the work of Hutchison (1), von Hösslin (2), Maver and Schwartz (3), and Peabody (4); more recently in the studies of Haden (5), Peters, Bulger, Eisenman, and Lee (6), and Sunderman, Austin, and Camac (7). The serum chlorides are greatly reduced and in spite of numerous investigations no agreement has been reached as to the cause therefor, nor has tissue analysis succeeded in revealing whither the lost chlorides disappear.

The fact that the blood chloride level is lowered in some other acute infectious diseases besides pneumonia has led to the suggestion that fever is in some manner responsible for the change (8, 9). Others have attributed it to the chemical changes resulting from protein destruction analogous to the chloride loss which occurs in intestinal obstruction (10), extensive burns (11), or after injections of histamine (12). The suggestion has been made that the reduction in chlorides is related to anoxemia (13).

The complexity of the situation in pneumonia with the concomitant occurrence of fever, tissue autolysis, and anoxemia makes an analysis of the problem exceedingly puzzling. For this reason we have attempted a simplification of it by experiments in animals with the hope of discovering whether changes in the environment, internal or external, would result in a diminution in the blood chlorides. Most of our experiments yielded negative results.

TABLE I.

The Effect of Experimental Pneumococcus Infection on the Serum Chlorides of Dogs.

Dog No.	Date	Time	Serum chlorides per liter	Body temperature
4	2/16/28	11:30 a.m.	mm 111.6	°C.
		11:45 a.m. inoculated by intrabronchial insufflation		
	2/16/28	4:35 p.m.	110.0	
	2/17/28	11:00 a.m. Survived	110.0	
5	2/18/28	10:25 a.m.	107.3	
		11:00 a.m. inoculated by intrabronchial insufflation		
	2/20/28	11:25 a.m.	105.8	
	2/21/28	4:28 p.m. Survived	113.0	
6	2/18/28	10:50 a.m.	110.3	
		11:30 a.m. inoculated by intrabronchial insufflation		
	2/20/28	11:30 a.m.	102.6	
	2/21/28	4:28 p.m. Survived	112.2	
7	2/23/28	3:25 p.m.	114.6	
		3:30 p.m. injected trans-pleurally		
	2/24/28	3:00 (?) p.m.	108.6	
	2/25/28	11:30 a.m.	109.5	
	2/27/28	Died		

TABLE I—*Concluded.*

Dog No.	Date	Time	Serum chlorides per liter	Body temperature
			<i>mm</i>	°C.
8	2/23/28	3:45 p.m.	110.1	
		3:50 p.m. injected trans- pleurally		
	2/24/28		107.5	40.2
	2/25/28	11:30 a.m.	103.6	40.1
	2/27/28	4:45 p.m.	97.6	40.5
	2/28/28		100.2	38.7
	2/29/28		103.9	39.3
	3/ 6/28	1:30 p.m. Survived	107.6	38.4

EXPERIMENTAL.

I. Blood Chlorides in Dogs before and after Pneumococcus Infection.

In three dogs, Nos. 4, 5, and 6, Table I, cultures of mouse-virulent pneumococci were insufflated intrabronchially according to the method of Lamar and Meltzer (14). The chlorides of the serum were analyzed by the method of Van Slyke (15) before inoculation and at intervals thereafter. None of the dogs reacted very severely to the injection and none developed a real pneumonia consolidation. In only one (No. 6) was there a chloride drop which appeared to be significant, amounting to 6.8 per cent in 48 hours.

In two dogs the pneumococcus cultures were injected transpleurally, resulting in one instance in death with a copious pleural exudate. The exudate gave a pure pneumococcus culture of the type injected. This dog (No. 7, Table I) showed a chloride drop amounting to approximately 5 per cent. The companion dog (No. 8, Table I), which survived the injection, showed a still greater reduction, reaching 11 per cent after 4 days.

It seems true, then, that a pneumococcus infection in dogs even without a pneumonic lesion may result in a lowering of blood chlorides.

II. The Influence of Fasting.

To control the effect of loss of appetite and consequent fasting in the infected animals, food was withheld from two normal dogs for a period of 5 days. Water was withheld only during the last 24 hours of the fasting period. The diminution in serum chloride concentration in one dog (No. 9) amounted to little more than 1 millimol per liter; from 112.5 to 111.1. The second animal (Dog. 10) showed a drop of 3.5 millimols, from 113.0 to 109.5. This does not correspond in order of magnitude to the change which may occur in pneumococcus infection.

III. The Factor of Anoxemia.

Five patients suffering from pneumonia, in whom arterial blood analysis demonstrated the presence of anoxemia associated with a reduced chloride concentration did not furnish evidence of a restoration of the chlorides to their normal levels when the anoxemia was relieved by placing the patients in an oxygen chamber. This led us to believe that the chloride loss was probably not related to oxygen want, as has been suggested (13). Nevertheless, to settle the point we produced a condition of acute anoxemia in a dog anesthetized by the intravenous injection of barbital-sodium.

The animal was tracheotomized and allowed to breathe, first room air, then a mixture containing 10 per cent oxygen and 90 per cent nitrogen, and finally, after breathing this mixture for 1 hour, room air was supplied instead. The arterial blood was analyzed for its oxygen and chloride content. During the hour of breathing the oxygen-poor gas mixture the per cent saturation fell from 88.8 to 59.4, whereas the chlorides fell 2 millimols. On readministering room air the per cent saturation rose to 91.4 and the chloride concentration increased only 0.7 millimol, as shown in Table II.

It can be concluded that acute anoxemia experimentally produced is not associated with any considerable alteration in the serum chloride level.

IV. The Effect of Tissue Destruction.

The possible relation between the presence in the body of products of tissue destruction and the reduction in serum chlorides has been mentioned. In this connection we recalled the chloride loss which occurs in association with intestinal obstruction (10), extensive skin

burns (11), and after histamine injection (12). The pneumonic process in the lung may, perhaps, represent another such process of tissue autolysis. To simulate such a reaction, we resorted to two methods; burning the skin and the production of experimental shock

TABLE II.

The Effect of Experimental Anoxemia on the Serum Chlorides of Dogs.

Time	Procedure	Gas breathed	Arterial O ₂ content	Arterial blood O ₂ capacity	Oxygen saturation	Serum chlorides per liter
			<i>mM</i>	<i>mM</i>	<i>per cent</i>	<i>mM</i>
11:29	1 hr. after an- esthesia Tracheotomy	Air	9.14	10.77	84.4	108.3
12:09						
1:20		Air	9.80	11.03	88.8	105.5
1:21		10% O ₂				
2:21		10% O ₂	6.72	11.32	59.4	103.5
2:25		Room air				
3:20		Room air	9.78	10.70	91.4	104.2

TABLE III.

The Effect on Serum Chlorides Produced by Clamping the Abdominal Aorta.

Time	Procedure	Serum chlorides per liter
		<i>mM</i>
10:00-10:30	Anesthetized with barbital-sodium	
12:31		109.5
1:17-2:24	Laparotomy; loose ligature about ab- dominal aorta; abdomen closed	
2:24		106.5
2:30	Aorta clamped Clamp released	
4:00		
4:15		111.5
4:45		106.6

through muscle anemia or muscle trauma (16), thinking that this might liberate a histamine-like substance and result in a chloride drop.

In one animal, Table III, under barbital anesthesia, the abdominal aorta was clamped for 1½ hours and blood was drawn for chloride analysis 15 minutes and

45 minutes after releasing the clamp. There was a slight rise in chloride concentration in the 15 minute sample, which fell again after 45 minutes to the level prevailing immediately before clamping. In another deeply anesthetized dog the leg muscles were crushed but the chloride concentration remained practically constant for 4 hours following the operation. The maximum drop was 1.1 millimols.

Davidson (11) has shown that in human beings extensive skin burns may be accompanied by a disappearance of the urinary chlorides and a marked drop in the level of the serum chlorides. In our experiments the skin of one animal under anesthesia with a large dose of barbital was extensively burned on both sides of the thorax. A little over an hour after the burn the serum chlorides had fallen 2.6 millimols. This level was maintained for the subsequent 12 hours.

V. The Effect of Anaphylactic Shock.

The relationship which is believed to exist between some acute infectious processes such as rheumatic fever, in which low serum chlorides may be found, and allergic states (17) led us to try the effect of anaphylactic shock on the serum chloride level.

A dog was sensitized according to the method described by Weil and Torrey (18) by the subcutaneous injection of 5 cc. of sterile horse serum. Immediately before injection a sample of venous blood was drawn for chloride analysis. The concentration was 106.1 millimols per liter. 19 days later another blood sample was drawn, which showed 104.4 millimols per liter. The dog was then given 20 cc. of horse serum intravenously. Within 2½ minutes it manifested the typical symptoms of acute anaphylaxis. 30 minutes after the injection while anaphylactic symptoms were still present a sample of blood drawn by cardiac puncture showed a slight rise in chloride concentration to 107.1 millimols. Two more specimens of blood, taken 2 hours and 5½ hours after the serum dose, showed the chloride level to be respectively 105.4 and 110 millimols per liter. Since the original level lay between 106.1 and 104.4, it cannot be said that any reduction had occurred.

VI. The Effect of Experimental Leucocytosis.

To see whether the low serum chloride concentration was, perhaps, related to the mobilization of the white blood corpuscles, an artificial leucocytosis was induced in a dog by the intramuscular injection of 5 gm. of sodium nucleinate dissolved in 40 cc. of distilled water.

At 11:45 a.m. the dog's leucocyte count was 10,500 per c. mm., the chloride concentration was 109.0 millimols per liter. The animal's rectal temperature was

39.3°C. At 12:30 the sodium nucleinate was injected. 2 hours later the animal looked ill, the temperature having risen to 40.4°C. 5½ hours after injection the white count had more than doubled, 26,200, and the next morning it was 22,600. At this time the temperature was 39.6°C. and the animal looked well. The chloride concentration was 108.1 millimols per liter. The next day the chloride concentration was 110.3 millimols.

Experimental leucocytosis from sodium nucleinate injection did not elicit any change in the serum chloride level.

VII. The Influence of Fever.

The simultaneous occurrence of low serum chlorides and an elevation of body temperature has already been referred to. It has been suggested by some investigators that the reduction in chlorides is, indeed, the result of the febrile state (18). Haldane's interesting observations on the water poisoning of miners is a related phenomenon (19). Apparently in this condition the exposure to high atmospheric temperatures results in so much loss of chloride in the sweat that chloride elimination in the urine may be entirely suspended. The great consumption of water by the thirsty miners so unbalances the ionic equilibrium in their blood that serious symptoms may result. Haldane found that the symptoms could be averted by allowing the miners to drink a ½ per cent solution of sodium chloride. It is unlikely that any such vicarious chloride loss occurs in pneumonia. Evidence points to the retention of chlorides in the tissues rather than to an increased elimination through portals other than the kidney. The suggestion that the reduction in chlorides is the result of blood dilution awaits experimental proof. Such a suggestion is, however, consistent with the hydremia which is said to occur during elevation of body temperature.

To produce fever in animals, we used two methods: the intravenous injection of methylene blue (20); and the application of the so called diathermy current to the body (21). Both result in a rapid rise of body temperature. The second method has the advantage of permitting the degree and rate of the rise to be accurately controlled. Neither method produced a drop in the serum chloride level.

A male mongrel weighing 11.2 kilos was anesthetized by the intravenous injection of barbital-sodium. When completely insensitive, the right femoral

TABLE IV.
The Effect of Heat and Water Ingestion on Serum Chlorides and Their Relation to Serum Water Content and Chloride Output.

Dog No.	Procedure	Rectal temperature	Blood volume estimated from body weight	Serum chlorides		Serum total base		Serum water content per liter	Serum solids per liter	Urine volume output	Urine chlorides	
				Per liter	Change	Per liter	Change				Per liter	In volume urine excreted
		°C.	cc.	mM	mM	m-Eq.	m-Eq.	gm.	gm.	cc.	mM	mM
26A	Water 1 liter	38.2	1040	113.0		158.2		927.8	72.2			
		38.2		105.0	-8.0	151.0	-7.2	933.5	66.5			
26B	Water 1 liter Diathermy Cooling	37.2	1040	112.4		163.7		920.9	79.1			
		37.2		103.2	-9.2	154.0	-9.7	931.9	68.1	14.5	10.5	0.15
		37.6		101.0	-2.2	145.0	-9.0	931.6	68.4	86.0	6.5	0.56
		37.2		102.2	+1.2	149.0	+4.0	931.0	69.0	132.0	1.8	0.24
22	Sodium-veronal Diathermy Water 1 liter Water 250 cc. Cooling	37.9	1700	108.2								
		41.4		108.2	0					71.0	78.3	5.56
		42.0		101.2	-7.0					2.4	80.5	0.19
				100.5	-0.7					0	0	0
		37.5		98.6	-1.9					0.7	71.5	0.05

23	Sodium-veronal Diathermy	38.8 41.0 41.5	1025	107.0	+0.8			916.4	83.6	9.0	11.0	0.10
	Water 390 cc. Water 180 cc. Water 20 cc.	40.0		107.2	-0.6			918.4	81.6	8.3	19.0	0.15
24	Sodium-veronal Diathermy	37.5 42.0	1300	107.8	+4.0			897.0	103.0			
	Water 3 liters	41.8 41.5		111.8 106.2 104.8	-5.6 -1.4			899.8 904.8 900.4	100.2 95.2 99.6			
25	Sodium-veronal Water 1 liter	38.2 37.6	985	110.4	-7.2			928.6	71.4	21.8	180.8	3.94
	Diathermy	40.2		103.2	-0.4			937.8	62.2	173.0	17.5	3.02
	Water 1 liter	41.0		102.8	-4.2			934.5	65.5	14.0	28.3	0.40
27	Sodium-veronal Diathermy	37.6 41.3 41.0	846	98.6 109.6	+0.2			939.1	60.9			
	Water 846 cc.	41.0		109.4 113.2	-0.2 +3.8			926.9 922.8 920.9	73.1 77.2 79.1			
				113.4	+0.2			923.0	77.0			

artery and left femoral veins were cannulated. A sample of arterial blood was withdrawn for chloride analysis. This showed a concentration of 107.6 millimols per liter. A 5 per cent solution of methylene blue in distilled water was then slowly infused, 18 cc. being given in approximately 2 hours. During this interval the temperature rose from 38.4°C. to 40.3°C. Within the next 2 hours it rose another 3°C. Blood drawn at the end of the experimental period after a temperature of 40–43°C. had been maintained for over 2 hours showed no drop in chloride concentration, which remained at 108.9 millimols per liter.

Experiments of a similar character in which elevation of body temperature was produced by diathermy gave similar results. Serum was analyzed for chlorides by the method of Van Slyke (15), for total base by the method of Van Slyke, Hiller, and Berthelsen (22), and for water content by drying at 110°C. to constant weight. The results are shown in Table IV.

Dogs 22, 23, 24, and 27, Table IV, were anesthetized with barbital-sodium, the femoral artery was cannulated, and diathermy applied to the thorax. Although there was a rise in temperature in each case, varying from 1.3–4.5°C., the serum chlorides were unchanged except in No. 24, in which case they rose 4 millimols per liter. When these same animals were given large volumes of water by stomach tube, with continuation of diathermy, the serum chlorides dropped in one animal (No. 22, Table IV) as much as 7 millimols per liter in an hour. When the volume of water given was less than the equivalent of the blood volume of the animal the serum chlorides showed no significant change, as in No. 23, which showed a drop of only 0.6 millimol per liter. When water was given an hour before the diathermy, as in No. 25, the serum chlorides fell 7.2 millimols per liter as a result of the water alone. After diathermy there was a further decrease of only 0.4 millimol. After the administration of another liter of water the serum chlorides fell 4.2 millimols. The serum water content in these experiments generally showed a rise when the chlorides fell.

These experiments seem to point to the conclusion that the drop in serum chlorides is due entirely to the dilution of the serum by the water administered, and heat itself is without effect.

The effect of water ingestion on serum chlorides has been investigated by Underhill and Sallick (23). They found a drop in chlorides after ingestion of large volumes of water, equivalent to 8 times the blood volume of the dogs. They demonstrated a blood dilution by a fall in the hemoglobin. They attributed the fall in serum chlorides to increased chloride excretion.

We performed two dilution experiments.

In No. 26A, Table IV, the dog was given 1 liter of water without diathermy. In an hour the serum chlorides fell 8 millimols per liter, the serum total base fell 7.2 millimols per liter, and the water content of the serum increased in amount proportional to the decrease of chlorides and base. In No. 26B, Table IV, the same experiment was repeated with similar results, then diathermy was applied with only slight further changes in serum chlorides and water content, and the total base continued to drop, then rose slightly during the process of cooling.

The amount of chlorides eliminated by the kidneys during the experiments was in most cases less than 1 millimol, so that the drop in serum chlorides could not be accounted for by excretion. In two cases, Nos. 22 and 25, Table IV, when the excretion was greater than this, it still did not account for the change in the serum. In No. 22, when the excretion of chlorides was 5.56 millimols in an hour, the serum chlorides showed no change during diathermy, whereas when water was given the serum chlorides fell 7 millimols per liter with only 0.19 millimol excreted.

DISCUSSION.

The object of this research was to find an experimental procedure analogous, if possible, to the one which operates spontaneously in pneumonia by which the chloride level in the dog's serum could be rapidly lowered for the purpose of studying both the mechanism and the effect of a lowered chloride level. None of the methods that were tried resulted in a chloride change which we could regard as significant. The only exceptions to this statement were the cases of experimental pneumococcus infection in dogs. In these cases two animals showed a change apparently of the same order of magnitude as is seen in the blood following the ingestion of a large volume of water. This change was due, we believe, to blood dilution, and did not appear to be influenced by the temperature of the animal. Despite the negative character of our experimental results, we present them because of the perplexity and confusion of the problem.

SUMMARY AND CONCLUSIONS.

Attempts have been made in dogs to lower the serum chlorides by means of various procedures. Of these the withholding of food, anoxemia, tissue destruction, anaphylactic shock, leucocytosis, and fever yielded negative results. Ingestion of large amounts of water lowered the chloride level independently of body temperature. The chloride drop under these circumstances could not be accounted for by excretion and appeared to run parallel with an increase in the serum

water content. Experimental pneumococcus infection in two animals reduced the serum chloride concentration.

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REACTIONS OF RABBITS TO NON-HEMOLYTIC STREPTOCOCCI.

I. GENERAL TUBERCULIN-LIKE HYPERSENSITIVENESS, ALLERGY, OR HYPERERGY FOLLOWING THE SECONDARY REACTION.

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INTRODUCTION.

In previous communications (1) we reported the phenomenon of secondary reaction to certain strains of green streptococci. Briefly this consists of an inflammatory reaction which appears about the 8th to 10th day after intracutaneous inoculation of rabbits with these microorganisms and at a time after the primary reaction has receded. Because we were unable to obtain the reaction in any species of animal except the rabbit it was thought that it might be a hypersensitive reaction of the nature of the Arthus phenomenon; but a number of experiments designed to test this hypothesis failed to prove it (2). As many features of the secondary reaction recalled certain points in the type of allergy seen in tuberculous infection attempts were made to determine whether it might not represent an allergic reaction of this type. The object of this communication is to present the detailed evidence which indicates that after the development of the secondary reaction the animals have a type of hypersensitiveness which closely resembles so called tuberculin allergy.

Methods.

The tests used to prove that an animal was hypersensitive have been three: ophthalmic, cutaneous and lethal. The first two of these have proven the most useful because they could be repeatedly applied to the same animal and thus variations in its allergic state be roughly estimated.

Ophthalmic Reaction.—The reactivity of the eye was tested as follows: The eye was anesthetized by instilling a drop of 10 per cent cocaine into the conjunctival sac. After 5 minutes the upper quadrant of the cornea was lightly scarified with the point of a very sharp iridectomy knife so that two or three very superficial abrasions passed from the center of the cornea, upwards to within 2 or 3 mm. of the sclerocorneal junction; but care was taken not to touch the sclera. Then a drop of bacterial sediment was placed in the conjunctival sac with a pipette and the lid rubbed over the surface of the eyeball a few times. The bacterial sediment was a centrifugate of an 18-hour blood broth culture.

In a normal rabbit such an inoculation was followed by very little reaction; usually nothing could be seen except a very slight gray streak at the site of scarification. Occasionally there was slight injection of the ocular conjunctiva for a few millimeters above the sclerocorneal junction, which sometimes persisted for 48 hours, but as a rule, when present, disappeared after 1 day.

In an animal in which a secondary reaction had been induced by several fair sized intracutaneous inoculations of green streptococci an entirely different reaction to corneal inoculation often occurred. The day following inoculation there was a distinct injection of the scleral conjunctiva in the upper quadrant; this increased in intensity for 2 to 4 days, and at times was so marked that the white appearance of the sclera was practically obliterated. In marked reactions there was distinct congestion of the palpebral conjunctiva of the upper lid, and with the most intense reactions distinct edema of this conjunctiva. Simultaneously turbidity of the upper quadrant of the cornea often appeared. This at first was seen along the lines of scarification but later usually involved the whole quadrant; in very severe reactions it involved the whole upper half and even extended to the lower half of the cornea. Usually, however, when well developed it was most intense near the sclerocorneal junction on either side of the scarification, and diminished in intensity towards the center of the cornea. At times corneal turbidity did not appear for 2 or 3 days. The turbidity persisted for varying periods according to the degree of hypersensitiveness of the animal. When slight it was present for only 2 or 3 days; when marked it lasted for 10 to 15 days. Diminution in corneal turbidity was usually preceded by decrease in the conjunctival congestion. Then gradually the opacity of the cornea diminished until macroscopically only a faint scar remained at the site of instrumental traumatization. Before this occurred, however, a distinct vascular reaction was observed. A day or two after the appearance of turbidity loops of small blood vessels appeared at the upper pole of the cornea and gradually grew downwards towards its center, most marked along the line of scarification. This pannus was usually grossly visible for 1 to 3 days after corneal turbidity had diminished. If the rabbit remained hypersensitive and 1 or 2 months later the cornea was similarly scarified in another quadrant and reinoculated these loops of vessels in the upper quadrant became more quickly visible than following the first inoculation; they also appeared earlier than the pannus at the site of the second corneal inoculation. This demonstrates that although the vessels could no longer be

seen with the unaided eye they probably remained latent but ready to function when injury was inflicted on the eye later. Similar vascular latency was described by Lewis and Montgomery (3) in tuberculous infection of rabbits' eyes. Some injection of the upper portion of the iris was sometimes seen early, but if reaction of the iris was a feature of the process it was masked by the clouding of the overlying cornea. Gross outpouring of exudative cells into the aqueous humor to the extent that it became turbid practically never occurred.

In the presence of the most severe reactions there was some mucopurulent secretion in the conjunctival sac, occasionally so abundant that it glued the lids together. Films of this exudate showed an admixture of pus cells and a few desquamated epithelial cells of conjunctival origin; but bacteria were never recognizable. Cultures of the conjunctival secretion and mucopurulent exudate uniformly failed to demonstrate viable streptococci.

These reactions resembled those severe lesions induced in tuberculous animals by the instillation of tuberculin into the conjunctival sac, and recalled some of the severe reactions seen in tuberculous patients at the time when Calmette's reaction was widely used. The interesting point is that green streptococci are so innocuous, relatively speaking, when applied in this manner to a normal rabbit's eye, yet set up such severe inflammatory reactions in the eyes of animals which have been hypersensitive. Naturally the intensity of the response varied within fairly wide limits, doubtless due to varying degrees of the general state of hypersensitiveness of the animals. Detailed evidence concerning this point will be given later. It may be stated here, however, that control hypersensitive animals tested in the same manner with plain broth never gave a positive eye reaction.

Lethal Reaction.—It was early noted that after a rabbit had shown a secondary reaction attempts to immunize it by intravenous injection of homologous streptococci in doses well tolerated by normal rabbits often resulted fatally. The deaths occurred between 18 and 48 hours following inoculation. Autopsy revealed a fairly constant gross alteration in certain organs: the lymph nodes were much enlarged and showed both over their surfaces and throughout their substance many hemorrhagic areas from pin-point to 1 or 2 mm. in diameter. The lymphatic tissue of the intestines was swollen and often showed hemorrhages. The thymus was enlarged from a normal of about 1 gm. to 3 or 4 gm. in weight, had a reddish gray edematous appearance and was filled with red punctate areas similar to those seen in the lymph nodes. The

marrow in the shafts of the long bones had a similar hemorrhagic appearance. While it was evident that the tissues of the hematopoietic system suffered severely in this reaction, the spleen, as a rule, did not show much gross evidence of damage. Although at times small focal hemorrhages were seen in the pericardium, endocardium and myocardium, visceral hemorrhages were rare compared with the frequency of those in the lymphatic tissues.

After the lethal test was applied more systematically it was noted that animals would sometimes appear very sick for 24 or 48 hours and then recover. If these animals were chloroformed on the 3rd or 4th day, similar, but less intense, gross pathological changes were seen. Normal rabbits and most animals that had not shown secondary reactions, on the other hand, manifested little, if any, clinical reaction to the usual test dose of streptococci, *i.e.*, the centrifugate of 4 cc. of broth culture per kilo body weight given intravenously, and if sacrificed the 3rd or 4th day by chloroforming, had little if any gross alteration of their lymphatic system. Occasionally animals which had previously failed to show secondary reactions at the site of their primary inoculations, succumbed to intravenous inoculation and showed characteristic postmortem alterations. The correlation of this lethal reaction with secondary reactions will be shown later.

Cutaneous Reaction.—Still another evidence of the altered reactivity of rabbits was furnished by reinoculating the skin with small doses of bacteria, first, doses that gave reactions in normal animals, and second, those that failed to elicit any gross reactions in normal rabbits. In the first instance it was observed that rabbits gave much more marked reactions at the time of second inoculation than originally, and also more intense lesions than normals simultaneously inoculated with comparable doses of the same culture. This is well brought out in the following experiment.¹

¹ The choosing of proper rabbits and preparation of their skin for testing was carried out in the following manner.

It was necessary to select the animals carefully because certain of them had pigmented skins and also areas in which the hair was coarse. This selection was easily accomplished by grasping the rabbit with the two hands and blowing gently from the thigh forward to the shoulder. In this manner the areas of pigmented skin were noted. These latter areas were found to be covered by a coarser hair.

Experiment 1.—A series of rabbits were inoculated intracutaneously with the sediment of 24-hour blood broth culture of Strain V92/0/10. Group A received 4 injections of 5 cc. each, Group B, 2 of 5 cc. each and Group C, 1 of 5 cc., and

TABLE I.
Average of Sum of Diameters of Lesions Following Reinoculation.

	Animals with previous secondary reaction				Animals without previous secondary reaction			
	Number of animals	Amount of inoculum			Number of animals	Amount of inoculum		
		10 ⁻¹ cc.	10 ⁻² cc.	10 ⁻³ cc.		10 ⁻¹ cc.	10 ⁻² cc.	10 ⁻³ cc.
Group A.....	5	67	37	28	1	62	37	25
“ B.....	5	71	34	22	—	—	—	—
“ C.....	5	54	32	20	1	44	24	18
Controls.....	3	39	20	9	—	—	—	—

Figures indicate average sum of diameters in millimeters.

all were observed daily for secondary reactions. 16 days later all were inoculated in 3 areas with the sediment of 10⁻¹ cc., 10⁻² cc. and 10⁻³ cc. of homologous culture respectively. At the same time 3 normal controls were inoculated similarly.

For the actual removal of the hair one required *impure* barium sulfide; it would not work if chemically pure. We have used Mallinckrodt's Barium Sulphide, Gray, Approx. 65 per cent. After wetting the hair thoroughly with water a fairly heavy coating of this powder was dusted on to the side of the rabbit. A heavy cotton swab on a stiff wire or stick was made wet and rubbed gently over the powdered area for about 1 to 1½ minutes; in this way a thick paste was made in the fur. As soon as it was noted that the hair was coming away the rabbit was held under running water until the paste was carefully and completely washed off by gently rubbing with the swab during the washing. If the operation had been properly performed the hair usually came away cleanly. If all the areas were not depilated the process was repeated over these areas, but it was found that there was danger of burning if the reagent was allowed to touch the bare skin. When burning resulted it was usually due to too vigorous rubbing or to allowing the paste to remain on the skin too long. It was also necessary to exercise care in removing all the barium sulfide from the hair at the margins of the denuded area. Following the washing this area was dried by gently patting with a towel and the animal placed back in the cage. It was found better to make the depilation 1 to 3 days before using the animals, for at the end of this period any burning of the skin could be detected and the areas avoided when applying the skin tests.

The maximum reaction occurred 48 hours after inoculation. Table I gives the average of the sum of the diameters of the lesions, measured at right angles to each other, recorded in the different groups at this time. One rabbit in each of Groups A and C had previously failed to show secondary reactions; and in these two the lesions resulting at the time of the second inoculation were slightly smaller than the average for their corresponding group. The figures in this table indicate clearly that the skin reactivity of the previously inoculated rabbits was from one and one-half to two times as intense as that of the controls. The animals of Groups A and B, which had received 4 and 2 lesions respectively, were more highly sensitive than those of Group C. Ophthalmic and lethal tests also demonstrated a similar high degree of sensitiveness of these two groups, compared with Group C, which in turn was more sensitive than the normal controls. In thickness and duration the lesions also showed striking differences. At the site of 10^{-3} cc. inoculations the most sensitive animals had indurated nodules from 2 to 2.5 mm. in height; the less sensitive animals had smaller lesions from 1 to 1.5 mm., and the controls had soft lesions 1 mm. or less. The average duration of these smallest lesions in the sensitive group was 8 days compared with 3 days in the controls. In still another respect the hypersensitive animals showed differences at the site of skin inoculation, *viz.* in color of the lesions, especially on the 2nd or 3rd day. Frequently the smaller lesions at this time had a dull red or dull pinkish red hue which did not disappear so readily on pressure as did the brighter red color seen in comparable lesions in normal animals. The color in these hypersensitive lesions reminds one somewhat of that seen in chronic granulomata such as lupus nodules or nodular syphilides.

In measuring the reactivity of the skin of rabbits with various doses of blood broth cultures of green streptococci it was observed that most normal rabbits gave no reaction with 10^{-4} cc. when this amount of culture was diluted with normal salt solution or plain broth and injected intracutaneously in quantities of 0.05 cc. On the other hand, animals previously injected intracutaneously with larger doses of culture showed lesions when tested later with these small quantities. Often the lesions in various animals had similar diameters, but those in the more sensitive animals were more raised, more indurated, duller red in color and persisted longer. It seemed, therefore, advisable to attempt to express the degree of reaction by the volume of the reacting macule, papule or nodule. The height of the lesion was estimated from measurements with special calipers. As the lesions were practically round, a rough estimation of their volume could be made by applying the formula $v = \frac{1}{6} \pi h(h^2 + 3a^2)$ where v = volume, h = height of lesion and $a = \frac{1}{2}$ of the average diameter. As a rule a was estimated

as $\frac{1}{4}$ of the sum of the two longest diameters taken at right angles to one another. From curves constructed from this formula for lesions of various sizes the volumes of measured lesions could be rapidly computed. It is recognized that a large source of error is inherent in this method; first because of the amount of reaction that may occur in the deeper layers of the skin without producing a comparable thickness of the papule; second, on curved surfaces such as presented by the contour of the rabbit's body it is difficult to estimate the height of the lesion accurately; and third, because different lesions on the same animal resulting from the same sized inoculum may vary in size,

TABLE II.

Correlation of Secondary Reactions, Ophthalmic Reactions and Test Lesions with 10^{-4} Cc.

Group	A	B	C	D	E (sick)
Secondary reactions.....	+	+	-	-	-
Ophthalmic reactions.....	\pm to +++	-	\pm	-	-
Number of animals.....	8	21	1	5	4
Average size of 10^{-4} cc. lesions, c. mm....	109	61	215	47	12
Above average.....	4	10	-	2	3
Variation in size, c. mm.....	110-180	65-120	-	75-92	13-20
Average.....	1	1	-	-	-
Below average.....	3	10	-	3	1
Variation in size, c. mm.....	54-72	25-48	-	23	0

- signifies negative reaction.

\pm to +++ signifies strength of reaction.

either because of differences of reactivity of skin of different textures and anatomical structure, or because the inoculum is not always introduced at the same depth. But in spite of these sources of error we have found this method of estimating the degree of sensitiveness to be of distinct value. The correlation of the intensity and frequency of the three reactions and their variation among rabbits is shown in Experiment 2.

Experiment 2.—Each of thirty-nine rabbits was inoculated intracutaneously with the sediment of 18-hour blood broth culture of Strain V110A/0/8 in 4 areas as follows: 2 with 5 cc., 1 with 10^{-1} cc. and 1 with 10^{-2} cc., and observed daily for

secondary reactions. 9 days later each animal was given a subcutaneous focus of agar inoculated with the sediment from 5 cc. of a similar culture. 38 days after the first inoculation the skins of all were tested with 10^{-4} cc. of homologous culture and the eye test was applied in the usual way. The results are shown in Table II.

It will be observed that the animals with positive ophthalmic reactions gave, on the average, the largest skin reactions. While twenty-nine animals had given secondary reactions about a month previous to the time of testing the skin and eye, only nine gave positive eye tests

TABLE III.

Correlation of Secondary Reaction, Ophthalmic Reaction and Skin Test Lesion with 10^{-4} Cc.

Group.....	A	B	C	D	E	F	G	H
Secondary reaction.....	+	+	±	—	±	+	±	—
Ophthalmic reaction.....	+	±	+	+	±	—	—	—
Number of animals.....	12	1	2	2	2	2	2	4
Average size of 10^{-4} cc. lesions, c. mm.....	88	65	55	29	20	22	33	22
Above average.....	4	...	1	1	1	1	1	2
Variation in size, c. mm.....	125-227	...	65	40	22	30	48	28-40
Average.....
Below average.....	8	...	1	1	1	1	1	2
Variation in size, c. mm.....	18-85	...	45	18	18	15	18	10-12

+ signifies distinctly positive secondary reaction.

± " doubtfully " " "

— " no secondary reaction.

... " no animals in this category.

on the 38th day. It was subsequently determined that the culture had deteriorated, which offered one possible explanation of this discrepancy. However, with the exception of the one animal in Group C, the rabbits with both positive secondary and ophthalmic reactions gave the average largest skin lesions, and those failing to give these reactions had the smallest; while those with a previously positive secondary reaction and later a negative ophthalmic reaction were midway between. The reactions in the four rabbits of Group E are noteworthy. These animals were very sick from snuffles or subcutaneous abscesses and gave

very poor reactions to all the tests. Their lesions probably represented the well known failure of cachectic individuals to react to infection in the normal manner.

The correlation of secondary reactions with ophthalmic reactions and skin reactivity to 10^{-4} cc. inoculation made at an earlier period than in Experiment 2 is shown in Experiment 3.

Experiment 3.—Twenty-seven rabbits were each inoculated intracutaneously in 4 areas with the sediment of 5 cc., 5 cc., 10^{-1} cc. and 10^{-4} cc. in 0.1 cc. amounts respectively; the inoculum was an 18-hour blood broth culture of Strain V110A/0/6 recently taken out of frozen and dried stock. None of the animals showed appreciable lesions at the site of the 10^{-4} cc. inoculation. On the 11th day the skin reactivity was tested with 10^{-4} cc. and the eye was tested with the same culture in the usual manner. The results of the three reactions are summarized in Table III.

Here again it will be seen that animals in Group A with both positive secondary and ophthalmic reactions gave on the average distinctly more voluminous skin reactions. One of this group had a lesion of only 18 c.mm., one a lesion of 40 c.mm., but in all of the others the lesions were 45 c.mm. or larger. Most of the other groups were too small to permit of valuable generalization; but among the twelve animals comprised in Groups D to H the average of the lesions was below 35 c.mm. and only one lesion as large as 48 c.mm. was found. Because these animals were to be used in other work it was not feasible to apply the lethal test.

An important point to be made from the results of both Experiments 2 and 3 is that an increased reactivity of the skin of rabbits follows previous intracutaneous inoculation whether or not a secondary reaction develops at the site of the primary inoculation. But in those animals which have recently shown a secondary reaction the reactivity of the skin to reinoculation as a rule is much greater than in those rabbits which had failed to show secondary reactions.

It is impossible to determine the degree of sensitiveness in a group of animals by applying all of the tests simultaneously, because a certain amount of time must be permitted to elapse in order to study the secondary reaction, the response to cutaneous reinoculation and the ophthalmic reaction before the lethal test can be applied. Animals vary considerably in the time at which they show a secondary reaction, and also in the degree of the reaction when it is present. All degrees of

intensity of ophthalmic reactions are also seen. A summary of the results obtained in 51 rabbits in three different experiments is shown in Table IV. All animals were sensitized with Strain V92/0/10. Six had 4 inoculations of sediment from 5 cc. each; 22 had 2 inoculations of 5 cc. each; 17 had 1 inoculation of 5 cc. and 1 of 10^{-1} cc.; and 6 had 1 of 5 cc. The ophthalmic test was applied on the 14th or 15th day and the lethal test on the 19th day in 17 animals, and on the 22nd day in the remainder. In this lot of animals no positive ophthalmic reactions were observed in those which had failed previously to show a secondary reaction. Among the total 42 animals comprised in Groups

TABLE IV.

Correlation of Occurrence of Secondary Reaction, Ophthalmic Reaction and Lethal Reaction.

Group.....	A		B		C	
Secondary reaction.....	+	+	+	+	-	-
Ophthalmic reaction.....	+	+	-	-	-	-
Lethal reaction.....	+	-	+	-	+	-
Number of animals.....	15	12	10	5	1	8
Percentage in group.....	55%	45%	66%	33%	11%	89%

+ signifies positive reaction.

- signifies negative reaction.

A and B, 25, or 60 per cent, died following intravenous inoculation, a striking contrast to the results in Group C. While a comparison of Groups A and B would indicate that rabbits with a positive ophthalmic reaction were less liable to succumb to intravenous inoculation than those without, this conclusion is probably not justified because of the differences in elapsed periods following the primary inoculation.

It is obvious that the development of a hypersensitive state as indicated by the occurrence of a secondary reaction cannot be detected unless the site of the primary injection can be observed and measured daily. The demonstration of ophthalmic hypersensitiveness and of the value of the lethal test, in animals made hypersensitive by intradermal inoculation, permitted us to apply these two tests to animals which were inoculated by various other routes. Experiments 5 and 6 give the results of such a study.

Experiment 5.—The animals were sensitized with the sediment of 10 cc. of 18-hour blood broth culture of Strain V92/0/11 which was concentrated by centrifugation and discarding the supernatant broth. The various sites of inoculation are indicated in Table V. In Group A the sediment of 5 cc. contained in 0.5 cc. solution was injected into each knee joint after we had assured ourselves that the needle was properly placed by first injecting and withdrawing normal salt solution. The subcutaneous injections were made over the large lumbar muscles; the intramuscular injections into these muscles. The intrapleural inoculum consisted of the sediment of 10 cc. of culture and was injected into the right pleural cavity of each animal; the intraperitoneal inoculum also consisted of the sediment of a similar amount. The paranasal sinuses were inoculated by etherizing the rabbits, boring a small hole through the thin plate of bone covering the sinuses, inserting a short needle through the trephine opening and scarifying the lining mucosa somewhat; then the sediment of 5 cc. of culture in 0.1 cc. volume was injected into the sinus on each side. The intratesticular inoculations were 0.1 cc. in volume. The vaginal inoculation was made by placing a purse string suture about the introitus, inserting a blunt needle and tightening the suture; 1 cc. of concentrated culture representing 10 cc. of growth was then injected and the needle withdrawn, and the suture tied. This suture was external to the urethra. The next day, both because the vulvæ were edematous and because the animals were unable to void, the sutures were cut and considerable amount of purulent exudate was discharged.

It was possible to measure the knees, subcutaneous lesions and testicles daily. The size and character of the lesions in the lumbar muscles could also be followed; hence in these four areas an increase in the intensity of the local reaction, about the usual time of occurrence of secondary reactions, could be estimated. But where the inoculations were made into body cavities no observations of this nature were possible.

Ophthalmic tests were applied to the right eye on the 14th day after the primary inoculation and to the left eye on the 20th day. The lethal test on the 24th consisted of 4 cc. of 18-hour blood broth culture per kilo body weight. 2 or 3 days later all animals which had not died as a result of this treatment were chloroformed and autopsied. It was thus possible to estimate roughly the sensitiveness of the tissues even though the animals had not been fatally infected.

It was evident from clinical observation that there often occurred an enlargement and more marked induration of the local inoculated areas about the 7th to 12th days, an indication that the reactivity of the tissues was undergoing an alteration at this time. Ophthalmic tests also indicated that animals in all groups were sensitized as a result of these inoculations. Twenty-one out of the thirty-six animals in the entire group gave a positive ophthalmic reaction; but comparison of

TABLE V.
Effect of Different Modes of Inoculation—into Tissues.

Group	Rabbit	Site of inoculation	Secondary reaction	Ophthalmic reaction		Lethal reaction 24th day	Thymus	Lymph nodes
				14th day	20th day			
A	Q571	Knee	±	+±D	++	—	—	—
	Q572		—	+	++	+	+++	+++
	Q573		+	++	++	—	—	—
	Q574		+++	—	—	—	—	—
	Q575		—	—	—	—	±	—
B	Q580	Subcutaneous	+++	—	—	—	—	—
	Q581		++	—	—	—	±	—
	Q582		++	++D	—	—	—	—
	Q583		+++	++D	++D	—	+	+
	Q584		—	—	—	—	±	±
C	Q585	Intramuscular	—	++	++	—	—	—
	Q586		?	—	—	—	—	—
	Q587		++	+D?	—	—	—	—
	Q588		—	+	++	—	±	—
	Q589		++	±D?	—	+	++	++
D	Q590	Intraperitoneal	0	±	—	—	—	—
	Q591		0	+±	—	—	—	—
	Q592		0	—	—	—	—	—
	Q593		0	+	+	+	+++	++
	Q594		0	±	—	—	±	—
E	Q595	Intrapleural	0	—	—	—	—	—
	Q596		0	+	±	—	—	—
	Q597		0	++	±	—	+	—
	Q598		0	—	—	—	±	—
	Q599		0	+±	—	—	±	—
	Q600		0	—	—	—	±	—
F	Q601	Paranasal sinus	0	+	+	—	+	—
	Q602		0	—	+±	—	+	—
	Q603		0	—	—	—	±	—
	Q604		0	+	+±	—	+	—
G	Q605	Intratesticular	?	+±±	—	—	+	—
	Q606		—	—	—	—	—	—
	Q607		—	—	—	—	—	—
	Q608		+	—	—	—	—	—
H	Q577	Vaginal	0	—	—	—	—	—
	Q578		0	++	+	—	—	—

± to +++ signifies various degrees of intensity of reaction.

— signifies negative reaction.

0 " no observation.

D " reactions delayed in time of appearance.

the corneal sensitiveness on the 14th and 20th days showed that at the time of first testing twenty of the animals showed positive reactions contrasted with only thirteen at the second testing; and in addition a number of the reactions at the time of later testing were distinctly weaker than on the 14th day. This indicates that in general the degree of sensitiveness was decreasing. It was previously reported by us (1) that the green streptococci are rapidly killed off in the intracutaneous foci. In this experiment one subcutaneous focus excised on the 11th day was sterile; and all foci cultured at autopsy failed to show living organisms. It thus seems probable that the stimulus to sensitization, which probably occurs as a result of reaction between tissues and bacteria, is not continuous because a new supply of bacteria is not available for its production. Likewise a diminution in the degree of hypersensitiveness probably explains the small number of lethal reactions, for this test was not applied until the 24th day after the primary inoculations.

A study of the various groups reveals some results of further interest. With the ophthalmic reaction as a guide, Group G with intratesticular inoculations and Group B, subcutaneously inoculated, showed the least sensitization, for in both the eye reactions were much delayed, and occurred relatively infrequently.

While a high proportion of the animals in Groups D and E, inoculated intraperitoneally and intrapleurally respectively, showed positive eye reactions on the 14th day, they were weak or delayed in three, and in all except Rabbit Q593 had become much weaker or negative when tested on the 20th day. This animal succumbed to intravenous inoculation 4 days later, an indication that its hypersensitiveness was continuing on a high level.

Group C, with intramuscular foci, had two members in which the eye reaction was weak and delayed on the 14th day, but also had two others in which the corneal sensitiveness persisted and was as strong or stronger on the 20th day. It is noteworthy that in only two other groups was this the case. In both the intraarticularly inoculated animals, Group A, and in those with paranasal sinus inoculations, Group F, the degree of corneal sensitization persisted and was as strong or stronger by the 20th day. It is interesting to note that infection of the mucosa of the paranasal sinuses without gross abscess formation,

as revealed post mortem, should lead to such rapid and continuing hypersensitiveness. A similar persisting state from intraarticular inoculation, is also interesting in view of the fact that in only one of these animals, A574, was there a frank abscess in the joint. In all others the synovia had a gelatinous appearance.

In the one rabbit with vaginal inoculation showing positive ophthalmic reaction there was an abscess in the vaginal wall.

TABLE VI.
Effect of Different Modes of Inoculation—Intravenous.

Group	Rabbit	Intravenous inoculation	Ophthalmic reaction		Lethal reaction 19th day	Thymus	Lymph nodes
			13th day	16th day			
		cc.					
A	Q388	10	×	—	—	—	—
	Q389	10	×	—	—	—	—
	Q390	10	×	—	—	—	—
B	Q382	10	—	—	—	—	—
	Q383	10	—	±?	—	—	—
	Q384	10	—	—	—	—	—
C	Q320	5, 10	×	×	10th day —	—	—
	Q321	5, 10	×	×	—	—	—
	Q322	5, 10	×	×	—	—	—

— signifies negative reaction.

× “ not inoculated at this time.

If now we contrast these results with those in a series of rabbits inoculated only intravenously, as in Experiment 6, the differences are most striking. The mode of inoculations and results are summarized in Table VI.

Experiment 6.—The animals of Groups A and B were inoculated intravenously with the sediment of 10 cc. of 20-hour blood broth culture of Strain V92/0/10; those of Group C with sediment of 5 cc. As we had occasionally observed deaths following early intravenous inoculation of intracutaneously sensitized animals the rabbits in Group C were reinoculated 3 days later with sediment of 10 cc. of culture. 2 days later Q321 died; but post mortem was found to have a severe *B. lepipecticum* infection; no gross lesions, however, were found in the thymus,

lymph nodes or bone marrow. The other two rabbits of Group C were again inoculated intravenously on the 10th day with sediment of 7.5 cc. of culture (4 cc. per kilo body weight) without any untoward symptoms, and with negative postmortem findings 3 days later.

The right eyes of Group B animals tested on the 13th day all failed to react. The left eyes of all surviving animals were tested on the 16th day with negative reactions in each instance except one where there was slight corneal turbidity, lasting only 2 days. Reactions of this degree sometimes occur in normal animals; hence it is safe to conclude that there was no corneal sensitization in any of the animals. All were inoculated intravenously with sediment of 10 cc. of homologous culture on the 19th day without showing any evidence of sickness afterwards. 3 days later, at autopsy, none showed any of the characteristic changes in the hematopoietic organs; Q390 showed a mild arthritis of the left knee and right shoulder. Thus lethal tests on the 4th, 10th and 19th days and ophthalmic tests on the 13th and 16th days failed to reveal any evidence of hypersensitiveness in animals in which the first inoculation was by the intravenous route and was of the same size as induced hypersensitiveness in all groups of animals in Experiment 5. Controls of the cultures used to make these tests proved that they were active in hypersensitive animals. Similar results following primary intravenous inoculations have been obtained with other strains of non-hemolytic streptococci.

It seems, therefore, that the condition necessary for the production of an early hypersensitive state, such as occurs in rabbits with the appearance of secondary reactions after intracutaneous inoculation with suitable non-hemolytic streptococci, is the production of an inflammatory focus some place in the body. Altered reaction doubtless occurs in an animal regardless of the microorganism introduced or the route through which it is introduced. There are, moreover, wide variations in the capacity of different animals to react to inoculation into similar tissues, and also distinct differences in different strains in their capacity to sensitize a group of animals. This has been already mentioned in our earlier publications, but the importance of recognizing variation in both the power of the microorganism to sensitize and the capacity of the animal to react is more clearly demonstrated in another place (4). The present discussion has rather to do with the influence of primary reaction in various tissues. When non-hemolytic streptococci of low virulence are introduced into the blood stream of normal animals they rapidly disappear and, in the doses here used, do not, as a rule, set up gross focal lesions in the tissues of the body. Their influence is probably exerted over a relatively wide area as

represented by the ramifications of the circulatory system. Doubtless certain organs bear the brunt of the attack more than others, but all are usually able to dispose of these streptococci without undergoing severe damage. When, on the other hand, these microorganisms are introduced directly into the tissues there is a local reaction, with a certain amount of tissue destruction, and before the body has been able to dispose of them effectively the inflammatory material has been produced either in sufficient quantity or of a quality to alter the bodily response in the direction of overreaction to a second inoculation at a distant site. It is interesting to note that such relatively slight reactions in the paranasal sinuses should lead to more marked sensitization than followed more marked reactions in the testicles, and also should be followed by corneal sensitization of longer duration than occurred after intrapleural or intraperitoneal inoculation. In view of the suspected rôle of streptococci in chronic arthritis the fact that general sensitization can be easily effected from intraarticular inoculation is also noteworthy.

DISCUSSION.

From the experiments presented in this and other communications it is obvious that the production of a lesion in rabbits by inoculation with non-hemolytic streptococci into some tissue is followed by a state of hypersensitiveness. This state is made evident by increased reactivity of the skin to reinoculation with small doses of the streptococci, by the marked reactivity of the scarified cornea—a non-vascular tissue—to instillation of the streptococci into the conjunctival sac and by the death of many of the animals following intravenous inoculation with cultures in amounts well tolerated by normal rabbits. The lesions found in the lymphatic and hematopoietic organs of these animals are grossly very similar to those described originally by Koch (5) in tuberculous animals following inoculations with large doses of tuberculin. We have observed similar lesions in normal rabbits following intravenous injections of more virulent hemolytic streptococci, and have noted ophthalmic reactions similar to those seen in hypersensitive rabbits following primary inoculation of the cornea of normal rabbits with living hemolytic streptococci. Thus, the condition of the hypersensitive rabbit has been altered in such a manner that the relatively

avirulent non-hemolytic streptococci set up immediate reactions grossly comparable to those which follow infection of normal animals with virulent hemolytic streptococci.

The death of these animals after intravenous inoculation does not occur immediately or within a period of 1 or 2 hours, nor is it accompanied by symptoms of acute shock such as were originally described by Arthus (6) in rabbits sensitized to various coagulable proteins. Neither did Auer (7) or Opie (8) describe lesions of this type in rabbits fatally shocked with horse serum or egg white. In a few animals intracutaneously inoculated with egg albumin we have not observed corneal hypersensitiveness comparable with that of the streptococcus-hypersensitive rabbits; nor have we found in the literature a description of this type of eye sensitiveness to non-bacterial coagulable proteins.

It has already been shown (2) that the secondary reaction bears no definite relationship to the presence of antibacterial immune bodies in the sera of the infected rabbits. In this respect the hypersensitiveness to non-hemolytic streptococci differs from that of rabbits immunized against non-bacterial coagulable proteins, where regardless of method of immunization a heightened antibody content of the serum is accompanied by an increased intensity of the Arthus phenomenon.

In all respects, therefore, where analogies can be applied, it seems that the hypersensitiveness of rabbits following the production of lesions with certain non-hemolytic streptococci, is comparable to that form described as tuberculin type of allergy or the hypersensitiveness of infection (Coca (9)). Zinsser (10) has clearly indicated how the two types of hypersensitiveness, or allergy, differ with respect to the presence of immune bodies; and later he expressed the opinion that the bacterial sensitization resulted from a peculiar form of autolysis of bacteria which probably occurs most readily in inflammatory foci. Roessle (11) also directed attention to the differences between the tissue reactions and incitants in the two types of hypersensitiveness.

It is, furthermore, perfectly evident that this type of hypersensitiveness or allergy is not peculiar to non-hemolytic streptococci, for in such chronic diseases as tuberculosis, syphilis, glanders, blastomycosis and trichophytosis, to mention only a few, skin reactions to the respective bacterial extracts are well known. Studies in typhoid reactions by

Gay (12) and his coworkers, and in reactivity to pneumococcus extracts or nucleoproteins after pneumococcus infections, by Mackenzie and Woo (13), and by Zinsser and Grinnell (14), show that acute infections also induce a similar type of bacterial allergy. Hanger's (15) studies of the reactions of rabbits to *B. lepi-septicum* filtrates also indicate that rabbits spontaneously infected with these microorganisms develop a peculiar type of allergy towards them.

In the field of streptococcus infections, especially as represented by recent developments in the study of scarlet fever and erysipelas, the accumulating evidence all indicates that bacterial allergy plays an important rôle in certain phases, at least, of these diseases. This work summarized by Zinsser (16) is well illustrated by the studies of Dochez and Stevens (17), Birkhaug (18), Zinsser and Grinnell (19) and Mackie and McLachlan (20); Kirchner (21) showed moreover that there was increasing hypersensitiveness of such an avascular tissue as the cornea to the so called scarlatinal toxin.

But it should be pointed out that in these studies although the primary inoculation in many instances was with cultures of the respective streptococci, in most instances the substance applied in testing was a broth filtrate or some bacterial fraction. In most of our studies we have used whole living non-hemolytic streptococci, though in a few instances we have tested hypersensitiveness with nucleoproteins, and filtrates. Mackenzie and Woo used nucleoproteins, and Zinsser and Grinnell used *Streptococcus scarlatinae* nucleoproteins as well as filtrates as testing substances. Still more recently Julianelle and Avery (22) have carried out more extensive series of experiments with pneumococcus infections in rabbits with results very similar to those we have obtained with non-hemolytic streptococci.

Because of the relatively low primary virulence of non-hemolytic streptococci we have thought it best to study the type of reaction following both primary inoculation and reinoculation with living microorganisms. The low invasive capacity of these streptococci and the rapidity with which they are annihilated in the rabbit's body made it especially desirable to determine just how the animal reacted towards the whole living microorganism during the various phases of immunity and resistance. While tubercle bacilli, *Spirochaeta pallida* or the various fungi found in trichophytosis have a comparable low grade of

primary virulence, the fact that they remain viable in the body of an animal and hence give rise to new foci for long periods creates a different set of conditions than follows inoculation with non-hemolytic streptococci. Primary inoculation with such microorganisms as capsulated pneumococci, hemolytic streptococci or staphylococci which have a higher invasive capacity, greater virulence and the ability to elaborate more markedly toxic substances, gives still other and more complicated conditions. Dold (23), though aware of the phenomenon of secondary reaction, did not record its occurrence in rabbits inoculated intracutaneously with a large variety of common pathogenic bacteria. Hence it appears that with this reaction of rabbits towards non-hemolytic streptococci we have available a set of factors quite favorable for analysis of certain phases of allergy and immunity.

The evidence brought out in Experiments 5 and 6 that foci some place in the body are necessary for the development of this type of allergy is most noteworthy. It is not merely a question of introducing the bacteria into the body but the fact that focal lesions are set up that seems to be important, for hypersensitiveness did not follow intravenous inoculation. Rapid destruction of the microorganisms without production of large focal lesions probably offers a suitable explanation of this phenomenon. If staphylococci, hemolytic streptococci or most strains of pneumococci, had been injected intravenously, either marked purulent focal lesions would have resulted, or the animal would have died before the allergic state could have been detected. Following intravenous injection with living tubercle bacilli there would have been produced multiple tubercles, foci of sufficient intensity to induce a condition of tuberculin allergy, and the persistence of tubercles would favor a continuance of the allergic state. In this connection, however, it is interesting to note that Petroff (24) observed that it was possible to induce a state of tuberculin allergy following inoculation of animals with killed tubercle bacilli by practically every route except intravenously. It is possible that the mode and duration of action of killed tubercle bacilli in respect of the inducing of allergy are much the same as those of non-hemolytic streptococci. The description by Calmette and Guérin (25) of secondary reactions at the site of inoculation of calves with attenuated tubercle bacilli B C G furnishes a striking parallel to our observations in rabbits. Wallgren (26), moreover, has very recently

reported the occurrence of secondary reactions in young children at the site of inoculation with Calmette's B C G bacilli, and found that cutaneous allergy to tuberculin only occurred in those children who had developed these secondary reactions.

We, therefore, seem to be in a position to give a reasonable explanation of the secondary reaction. Following the intracutaneous inoculation of rabbits with any strain of streptococcus there develops a state of tuberculin-like hypersensitiveness; but certain strains possess the capacity of stimulating the hypersensitiveness to a higher level than others, and certain rabbits are more capable of reacting, as is made evident by retesting the animals in different ways. Following the primary intradermal reaction there persists at these primary foci a certain amount of residual antigenic substance. When a sufficiently high degree of general hypersensitiveness develops the cells in the immediate vicinity of the primary lesion are in a condition to react with small amounts of suitable bacterial substances whether they are either freshly introduced or residual. The secondary reaction is, therefore, apparently an evidence of this reaction with some residual antigen persisting at the site of primary inoculation in an animal which has developed a general state of hypersensitiveness. The peculiar feature is that some strains should possess these stimulating or reacting substances to such a degree, while others are strains apparently lacking in them.

In the studies of Dochez and Stevens and of Birkhaug concerning the two phases of sensitization of rabbits towards toxic filtrates various routes of inoculation were employed. The first observers injected toxic filtrates intracutaneously, and later intravenously; the last experimenter injected living indifferent streptococci intraperitoneally. While Dochez and Stevens observed that the second phase of allergy followed prolonged intravenous inoculation, Birkhaug reported that skin desensitization followed intravenous injection of toxic filtrates. Therefore, there appears to be some contradictory evidence concerning the results following different routes of inoculation. But it should be noted that different types of streptococci were used by these two sets of workers. Nevertheless the influence of the types of lesions resulting from the different routes of inoculation has in general not been as carefully considered as it probably deserves. If our observations

concerning the effect of production of focal lesions compared with that of intravenous inoculation without focal lesions are substantiated with other bacteria, it may be necessary to reevaluate certain of the factors in the production of tuberculin-like bacterial allergy.

CONCLUSIONS.

1. Accompanying and following the evolution of a secondary reaction in the skin of rabbits after inoculation with suitable doses of certain non-hemolytic streptococci there quickly develops a general state of hypersensitiveness or allergy towards these streptococci.

2. This state is made evident by ophthalmic reactions following corneal inoculations, by much increased reactivity of the skin following intracutaneous reinoculations, and by lethal reactions, resembling tuberculin shock, following intravenous inoculations.

3. In a given hypersensitive rabbit there is a rough parallelism in the intensities of these different kinds of reactions.

4. This type of hypersensitiveness or bacterial allergy does not follow primary intravenous inoculation of rabbits with comparable doses of the streptococci employed.

5. As the development of this type of hypersensitiveness or bacterial allergy seems to accompany the production of focal lesions of a certain intensity, it is probable that in these foci are produced the substances or conditions which lead to this type of bacterial allergy.

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STUDIES ON INDIFFERENT STREPTOCOCCI.

III. THE ALLERGIZING CAPACITY OF DIFFERENT STRAINS.

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The discovery in this laboratory (1) of the secondary reaction which occurs at a definite interval following the intradermal inoculation into rabbits of properly chosen doses of certain strains of *Streptococcus viridans* has pointed the way for a number of investigations into the general field of bacterial allergy, with reference particularly to the streptococcus group (2). The results of these investigations, in conjunction with a number of findings in patients with rheumatic fever, have given rise to a working theory in which the phenomena of the disease are interpreted in terms of allergy or hyperergy to streptococci (3).¹ Most of the experimental data reported have been obtained with certain strains of *Streptococcus viridans*. In view, however, of the fact that the indifferent streptococci have been considered to play a part in the production of the disease (4), and in some quarters have been assigned an exclusive etiologic rôle, it has seemed desirable to investigate these organisms with a view to determining their potentialities as allergizing agents. A number of interesting observations along this line have been published recently by Birkhaug (5). His animals were inoculated intraperitoneally and were tested only with filtrates.

Inasmuch as in the case of *Streptococcus viridans* the capacity to produce the secondary reaction in rabbits had served as a fairly reliable index of the allergizing activity of given strains, the obvious approach to the problem was to observe the frequency with which this reaction occurred following the properly executed inoculation with various strains of indifferent streptococci. For this purpose a number of

¹ A similar theory was quite independently developed by Zinsser, *Bull. New York Acad. Med.*, 1928, iv, 351, and previously suggested in an article by him and Grinnell, *J. Immunol.*, 1925, x, 725.

cultures were selected from among those carried in stock at the time. Twenty-four examples of Type I and twenty of Group X (6) were studied; among the latter were included eleven which in our hands failed to ferment inulin. Some of the strains had been isolated from patients suffering from active rheumatic fever; others from persons in sound health who had never suffered from the disease; still others from hospital patients suffering from conditions unrelated to rheumatism.

TABLE I.

*Scheme for Inoculation of Rabbits
and Tabulation of Results.*

Rabbit No.			
1	A	B	C
2	B	C	D
3	C	D	E
4	D	E	F
5	E	F	A
6	F	A	B

Methods.

Inasmuch as only in the rabbit has the gross secondary reaction been observed, this was of necessity the experimental animal chosen. Inoculations were made into areas of unpigmented skin depilated at least 24 hours previously with a watery paste of commercial barium sulfide (Mallinckrodt). The bacteria were grown for 24 hours in phosphate broth, pH 7.8, containing 2 per cent of defibrinated rabbits' blood and 0.05 per cent of dextrose. When such cultures were administered in amounts less than 10^{-1} cc., dilutions were made in phosphate broth such that the dose desired was contained in a volume of 0.05 cc. As a rule each strain was administered in three doses, 10^{-1} cc., 10^{-2} cc. and 10^{-3} cc.; but in a few experiments the 10^{-1} cc. dose was omitted. Each rabbit was tested with three strains in the dosages given and each strain was tested in at least three animals.

By a method of staggering the inocula it was arranged that no two animals received the same three strains. Results were tabulated in accordance with this scheme, which is illustrated in Table I. Thus six strains, which might be designated A-F, would be injected into six rabbits, as indicated in Table I. Certain advantages accrue to this method of study, for both variation in reactivity of the rabbits and in allergizing capacity of the microorganisms are demonstrated. In the first place, a small number of animals are incompetent to develop the secondary reaction, whether through illness (cachectic response), or because of the existence of a special condition of natural immunity. On the other hand, many strains of indifferent streptococci, more particularly of Group X, are endowed with little or no allergizing capacity. The percentage of rabbits refractory to sensitization is so small that it is unlikely that more than one in four or five will be encountered in any single group. By reading horizontally it is a simple matter to determine the

presence of refractory animals, as well as of those unusually responsive to sensitization. By reading along the dotted diagonals the number and degree of the secondary reactions encountered will serve as a fair index to the sensitizing power

TABLE II.

Variations in Allergizing Capacity of Nine Strains of Indifferent Streptococci Tested in Nine Rabbits.

Rabbit No.	10^{-1}	10^{-2}	10^{-3}	10^{-1}	10^{-2}	10^{-3}	10^{-1}	10^{-2}	10^{-3}
1	+	±	+++	-	-	-	±	±	-
2	-	++	+++	-	++	+++	+	++	+++
3	+	+	-	+	+	-	-	±	-
4	+	±	?	-	-	+++	±?	-	-
5	+++	+++	++	-	-	-	+++	-	-
6	-	-	-	++	+++	-	-	-	-
7	-	-	-	-	-	-	-	++	-
8	-	-	-	++	+++	-	+++	++	+++
9	++	+++	-	+++	+++	+++	±	+	++

of the strains. The method is, of course, of less value when one is dealing with a number of strains, few or none of which possess more than a weak or irregular allergizing capacity. Under such circumstances it is desirable to introduce strains

of known potentialities as controls; in Table I Strains A and D might be thus chosen; the only alternative to this, in case of the development of a small percentage of secondary reactions, is repetition of the experiment.

The results of one among several experiments are shown in Table II. Nine rabbits were each inoculated in nine areas with doses and strains of different streptococci as indicated in the headings. All of the lesions were measured daily for 3 weeks. The scheme used in charting the intensity of the secondary reactions was as follows: the two diameters of the lesions were added, and from this result was subtracted the sum of the two diameters observed when the lesion was at its minimum preceding the development of the secondary reaction. When this difference amounted to 1-3 mm., the \pm sign was charted; 4-6 mm., +; 7-9 mm., ++; 10-12 mm., +++; 13 mm. or more, +++. The - sign indicates absence of secondary reaction.

TABLE III.
Intensity of Secondary Reactions from Type I Strains.

Total secondary reactions	Intensity				
	\pm	+	++	+++	++++
150	16 10.7%	26 17.3%	24 16.0%	13 8.7%	71 47.3%

For meaning of + and - signs, see text.

In this experiment Rabbits 1, 3, 4 and 7 were poorly adapted to the purpose in hand, as they were obviously in varying degree refractory to sensitization, No. 7 almost completely so. This unusually high proportion of unsatisfactory rabbits might have invalidated the experiment had it not been for the peculiar distribution of the strains. However, by reading along the diagonal lines and making allowances for the refractory rabbits as their levels are reached, it is made clear that Strains Q155, P71B, RF18, P59B, RF2, Q205 and RF8 really possess the allergizing power, while Strains P31 and P61 are devoid of it, for in areas inoculated with the latter microorganisms secondary reactions failed to appear even though the tests were made on such highly reactive animals as No. 8 and No. 5 respectively. Variations among the positively reacting microorganisms in capacity to stimulate secondary reactions were also made evident in this experiment. For example, Strain Q155 gave good, even though not marked, reactions in such a poorly reacting animal as No. 1; while Strain RF18 gave very poor reactions in this animal and in No. 3, but better reactions in the more reactive No. 2.

Ophthalmic tests were carried out as described elsewhere (3), by application of culture sediment to corneas previously scarified under novocaine anesthesia.

RESULTS.

With the 24 strains of Type I, 215 lesions were produced in 82 animals. In 150 (69.7 per cent) of these lesions secondary reactions appeared within 3 weeks following the date of inoculation, and usually before the 12th day.

In Table III the 150 secondary reactions have been analyzed with respect to their intensity, in accordance with the arbitrary formula

TABLE IV.

Incidence of Secondary Reactions in Lesions Resulting from Different Inocula of Type I Strains.

	Size of inocula		
	10 ⁻¹ cc.	10 ⁻² cc.	10 ⁻³ cc.
Total lesions.....	51	82	82
Secondary reactions.....	37 (72.6%)	69 (84.2%)	44 (53.7%)

TABLE V.

Intensity Distribution of Secondary Reactions among Different Inocula of Type I Strains.

	Size of inocula		
	10 ⁻¹ cc.	10 ⁻² cc.	10 ⁻³ cc.
Total secondary reactions.....	37	69	44
Intensity ±	4 (10.8%)	11 (15.9%)	1 (2.3%)
+	8 (21.6%)	15 (21.7%)	3 (6.8%)
++	12 (32.4%)	11 (15.9%)	1 (2.3%)
+++	2 (5.4%)	6 (8.7%)	5 (11.4%)
++++	11 (29.7%)	26 (37.7%)	34 (75.2%)

mentioned above. Nearly 50 per cent of the reactions were of sufficient degree to be expressed as ++++. In Table IV the 215 lesions produced by the Type I strains have been analyzed with respect to the occurrence of the secondary reaction in the lesions resulting from the various inocula. The high percentage (53.7 per cent) of secondary reactions in the 10⁻³ cc. lesions is noteworthy, and is in contrast to observations made with *Streptococcus viridans*; for we have repeatedly observed that secondary reactions in lesions resulting from inocula-

tion with 10^{-3} cc. of *Streptococcus viridans* cultures are relatively uncommon.

In Table V are analyzed the lesions resulting in secondary reactions with respect to their distribution by intensity among the three inocula. The tendency for the 10^{-3} cc. lesions to show more strongly marked reactions than the 10^{-1} cc. lesions is largely attributable to the method

TABLE VI.
Intensity of Secondary Reactions from Group X Strains.

	Total secondary reactions	Intensity				
		±	+	++	+++	++++
Inulin fermenters	41	9 21.9%	20 48.8%	6 14.6%	1 2.4%	5 12.2%
Non-fermenters	39	9 23.1%	11 28.2%	7 17.9%	0 —	12 30.8%

TABLE VII.
Relation between Size of Inoculum and Incidence of Secondary Reaction in Group X Lesions.

		Size of inocula		
		10^{-1} cc.	10^{-2} cc.	10^{-3} cc.
Inulin fermenters	Total lesions	23	23	23
	Secondary reactions	17 (74.0%)	16 (69.6%)	8 (34.8%)
Non-fermenters	Total lesions	25	34	34
	Secondary reactions	15 (60.0%)	16 (47.1%)	8 (23.5%)

of recording by diameters. The lesions produced by 10^{-1} cc. doses of Type I strains are relatively quite large. In the case of such large lesions the secondary reaction manifests itself as much by increase in volume as by lengthening of diameters, whereas smaller lesions frequently increase disproportionately in circumference. For this reason the data of Table IV are perhaps a truer picture of conditions than are those of Table V.

The twenty strains of Group X have been analyzed in two groups according to their capacity to ferment inulin in Hiss serum water tubes. With nine strains of inulin-fermenting organisms 69 lesions were produced in 23 animals. In 41 (59.4 per cent) of these lesions secondary reactions appeared within 2 weeks of the date of inoculation. Similarly, with the eleven non-fermenting strains 93 lesions were produced in 34 animals; in 39 (42 per cent) of these lesions secondary reactions appeared within the same period of time.

TABLE VIII.

Intensity Distribution of Secondary Reactions among Different Inocula of Group X Strains.

		Size of inocula		
		10 ⁻¹ cc.	10 ⁻² cc.	10 ⁻³ cc.
Total secondary reactions.....		17	16	8
Inulin fermenters	Intensity ±	4 (23.5%)	4 (25.0%)	1 (12.5%)
	+	11 (64.7%)	7 (43.7%)	2 (25.0%)
	++	2 (11.8%)	3 (18.7%)	1 (12.5%)
	+++	0 —	1 (6.3%)	0 —
	++++	0 —	1 (6.3%)	4 (50.0%)
Total secondary reactions.....		15	16	18 —
Non-fermenters	Intensity ±	4 (26.7%)	3 (18.7%)	2 (25.0%)
	+	4 (26.7%)	7 (43.7%)	0 —
	++	2 (13.3%)	2 (25.0%)	3 (37.5%)
	+++	0 —	0 —	0 —
	++++	5 (33.3%)	4 (50.0%)	3 (37.5%)

In Table VI these reactions are analyzed with respect to their intensity. Not only does the secondary reaction appear with less frequency in the lesions given by Group X strains, but comparison with Table III shows that there is a decided tendency for it to be less intense when present.

In Table VII the total Group X lesions have been analyzed with respect to the relation between size of inoculum and incidence of the secondary reaction. Whereas the incidence of the secondary reaction in the 10⁻¹ cc. lesions approaches that observed for the Type I strains, there is a decided tendency for it to be lower in the cases of the smaller lesions. This is obvious from comparison of Table VII with Table IV.

In Table VIII is given the intensity distribution of the secondary reactions among the lesions resulting from the different inocula of Group X strains.

DISCUSSION.

Even a cursory examination of Tables III to VIII will reveal the fact that the capacity to give rise to lesions in which a secondary reaction will appear is a fairly constant characteristic of the indifferent streptococci. In the case of *Streptococcus viridans* only strains derived from patients suffering from active disease of some sort were found to possess this capacity, while strains saprophytic in the respiratory passages were devoid of it (1), but this point was not investigated so extensively as in the present study. As regards the indifferent streptococci, however, the source of the strains has been immaterial; certain cultures isolated from the throats of normal individuals have given rise to secondary reactions as frequently and in as great intensity as have any of those derived from patients with manifestations of active rheumatic fever.

The capacity to yield the secondary reaction is greater for the strains of Type I than for those of Group X; and within the latter group the inulin-fermenting organisms show it more frequently than do those which fail to ferment this carbohydrate. In the case of the latter organisms it is found that the 10^{-1} cc. lesions do not yield the secondary reaction any more frequently than do the 10^{-3} cc. lesions of the Type I strains. Furthermore, examination of the tables reveals that in lesions resulting from 10^{-3} cc. inocula there is a disproportionate drop in the percentage of secondary reaction in passing from Type I to the non-fermenting members of Group X. A possible explanation for this is found in the comparison of the primary reactions. The lesions resulting from the inoculation of 10^{-1} cc. of Type I culture into a normal animal are comparatively large, measuring frequently 30 mm. or more lengthwise by 15–20 mm. in breadth, and often 3–4 mm. in thickness. They are deeply congested and considerably infiltrated, and are usually surrounded by an area of slight edema. The 10^{-3} cc. lesions are at first at least 1 mm. in thickness, and remain measurable usually for 4 to 5 days. As a rule it may be said that the strains of Group X produce smaller primary lesions, and that the smallest of all

are given by those which fail to ferment inulin. Examples of the latter have been encountered, 10^{-1} cc. of which failed to cause a lesion larger than that produced by 10^{-3} cc. of a Type I strain. These differences are too great to be explainable upon the basis of growth inequalities, and would appear rather to indicate that the Type I organisms possess primarily a greater toxicity for tissue. Either upon this or some similar factor, or upon the relative size of the primary lesion, or upon both combined, the appearance of the secondary reaction would seem to depend.

The above statements must be understood to refer to general trends. Specifically, a certain amount of variability has been found in the capacity of the strains of any one group to yield the secondary reaction. It affects the organisms of Type I, however, less than those of Group X. The reasons for this variability are not known. It seems to be independent of the source of the culture, its luxuriance of growth, or possibly its capacity for toxin production.

The recognition of variability in two factors, *viz.*, in the allergizing capacity of different strains of streptococci and in the different reacting capacity of rabbits is most important, not only from the viewpoint of simple experimentation, but from that of infection and immunity. As is indicated elsewhere (7) the degree of reactivity to reinfection is conditioned to a considerable extent by the type of response the animal exhibits following the primary infection; hence if a poorly reacting animal is infected with a strongly allergizing strain its subsequent reactivity may be similar to that of a strongly reacting animal primarily infected with a weakly allergizing strain. When, on the other hand, both parasite and host possess in a high degree the substances or qualities favorable for production of hypersensitiveness, subsequent reinfections will stimulate the most marked response; and finally if neither possesses these qualities the minimum of effect from subsequent reinfections can be anticipated. It is, of course, well recognized that these two factors may, and probably do, play an important rôle in the infectious diseases; but such variability in the two elements, host and parasite, are rarely so readily demonstrable as in these experiments.

This point is further emphasized by comparison of certain indifferent and green streptococci.

Positive ophthalmic tests (3) are readily obtainable in animals properly sensitized by the intradermal route with Type I strains. They have frequently been observed following a single series of injections, the total dosage of which has not exceeded 0.333 cc. of Type I culture. The production of a comparable degree of hyperergy with *Streptococcus viridans* requires a definitely greater initial dosage of culture, and, unless this dosage is rather large, a somewhat more protracted period of sensitization. The indifferent streptococci of Type I are therefore rather more efficient allergizing organisms than are those of the *viridans* group, in that smaller intradermal dosages will give rise in a shorter time to a demonstrable condition of hyperergy.

A positive lethal test (3), while not obtainable with the same constancy as the ophthalmic reaction, is occasionally encountered even with animals sensitized by a single series of intradermal injections of Type I strains, the total dosage of which does not exceed 0.333 cc. At autopsy the findings are similar to those which have been described in previous reports (3) dealing with *Streptococcus viridans*; namely, enlarged and congested lymph nodes and thymus with hemorrhages in these organs, in the bone marrow and elsewhere. In addition, striking gross edema and congestion of the lungs are encountered. These findings differ radically from those which follow the intravenous injection of the organisms into normal animals; in the latter case, death occurs only after a period of several weeks, and the autopsy reveals the lesions of vegetative endocarditis which have been described by other observers (4).

SUMMARY AND CONCLUSIONS.

The indifferent streptococci are remarkably efficient allergizing agents when inoculated intradermally into rabbits.

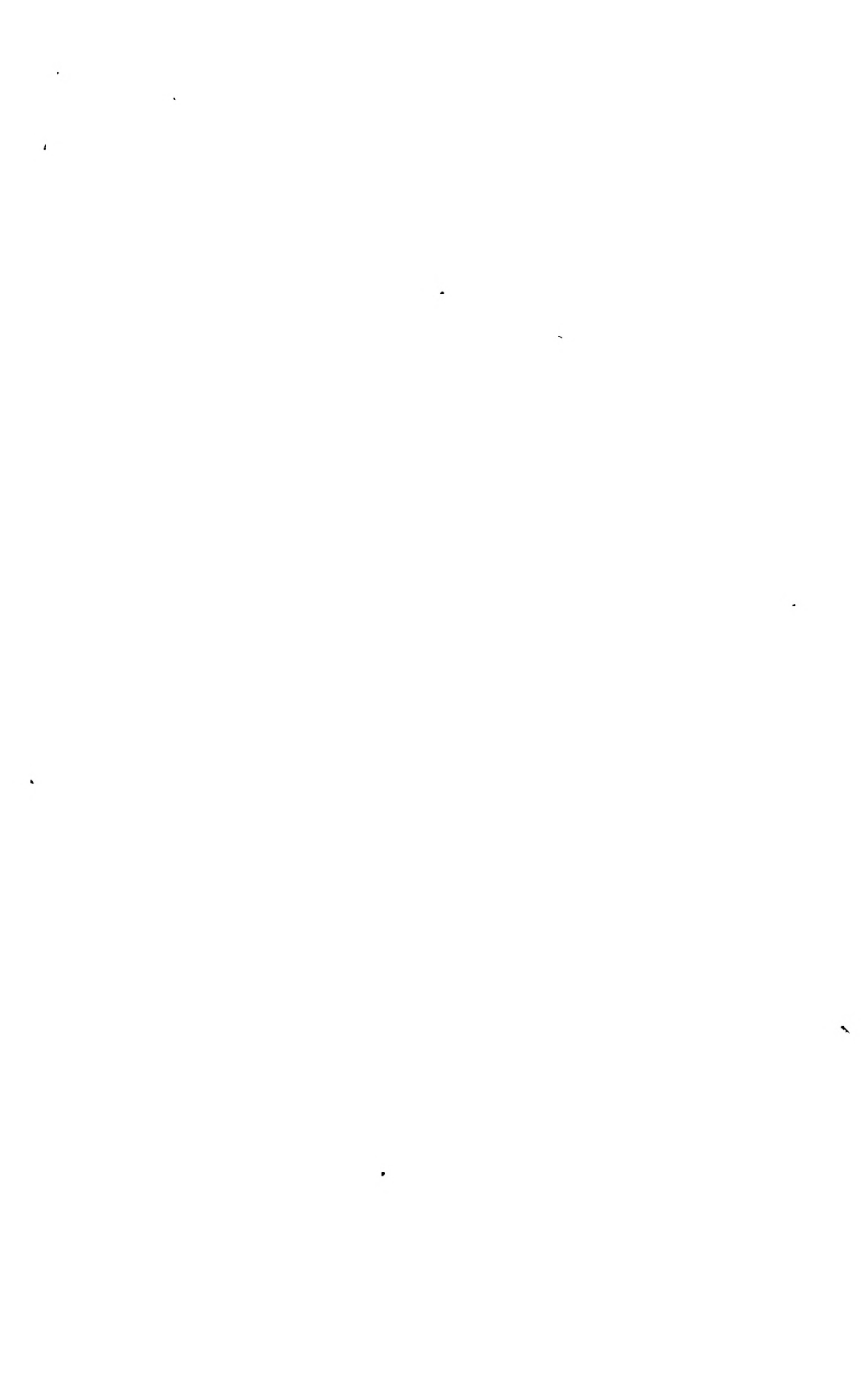
This is revealed by the high percentage of secondary reactions which occur in the lesions resulting from the inoculation of small doses of these organisms, and by the relative frequency with which positive ophthalmic reactions are obtained following sensitization with relatively small doses.

This allergizing capacity is most marked in the organisms of Type I and least marked in the non-inulin-fermenting strains of Group X.

The different resultants emerging from variations in allergizing capacity of streptococci and reactivity of host are clearly demonstrated in this series of experiments.

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TRANSMISSION OF FOWL-POX BY MOSQUITOES.

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PLATES 38 to 41.

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Fowl-pox, a common disease of the barnyard, annually recurs in epidemic form throughout numerous countries. The virus that produces the disease is an infectious agent (1) capable of passing through bacteria-tight filters (2), and, though not identical with other pox-producing viruses, probably is closely related to some of them. As a rule fowl-pox is not extremely fatal, yet the depression in the egg-laying activity of infected fowls leads to a great economic loss.

From the records of the epidemics of fowl-pox studied by Bollinger (1), Sanfelice (3), and others, it appears that the disease occurs for the most part during the spring and fall months. Summer epidemics, however, have been observed. In America, according to Beaudette (4), the disease is most prevalent during April and October.

Although the fact that fowl-pox is transmissible by direct inoculation (1) has long been recognized, the mode of its rapid spread under natural conditions has remained somewhat of a mystery. Contact infection undoubtedly can occur at times. Bollinger (1) reported that he obtained an infection in a healthy chicken by placing it in a yard with a sick fowl. Burnet (5), however, was able to obtain results similar to Bollinger's only when the majority of the feathers had been plucked from the healthy fowl before it was placed in contact with the diseased one. He was also able to infect a pigeon by feeding it virus, but, in order to assure a positive result, he mixed ground glass with the meal of wheat and virus. In this instance the lesions occurred in the mouth and esophagus and not on the comb, the usual place for the disease to manifest itself. Notwithstanding the results above described, the experience of most workers (Burnet (5)) has been that under laboratory conditions healthy fowls housed in the same cage with diseased birds do not contract fowl-pox. Indeed, Goodpasture (6) states that no method of artificial infection seems adequate to explain the rapid and thorough infestations which occur under natural conditions.

Some workers have attempted to incriminate (7) various insects as vectors of fowl-pox. Mégnin (8) suggests that flies and other insects may play a rôle in the dissemination of the disease, but offers no convincing experimental evidence

in support of his hypothesis. Farmers (4, 9) in various localities are of the opinion that mosquitoes are instrumental in the spread of the malady, but no records of experiments substantiating the idea have been found. Schuberg and Kuhn (10), however, report that in each of 3 tests they succeeded in transferring fowl-pox from diseased to normal chickens by means of interrupted feedings of *Stomoxys calcitrans*,—the flies starting their meal on an infected bird and completing it on a healthy one. These experiments were not extended, and it appears that they have aroused little, if any, interest.

The records of failures to transmit fowl-pox under the conditions indicated above, the occurrence of epidemics in the spring, summer, and fall, the fact that the lesions appear on exposed parts of the body (comb and wattles), and the experiments reported by Schuberg and Kuhn (10) indicated to us that biting insects may play an important rôle in the transmission of the disease. The experiments described in the present paper were undertaken to ascertain whether mosquitoes are of any significance in the spread of fowl-pox.

EXPERIMENTAL.

Methods and Materials.

Virus.—The strain of fowl-pox virus employed was originally obtained from Dr. Andervont and has been in this laboratory for approximately 2 years. Before starting our experiments we tested the activity of the virus by means of 2 serial passages in chickens, and also proved its purity by suitable tests in rabbits.

Chickens.—With a few exceptions the chickens employed in the experiments were White Leghorns. In working with fowl-pox one must remember that it is a natural disease of chickens and that immune or partially immune fowls are likely to be encountered. Consequently experiments should be repeated frequently or should be run in duplicate.

Mosquitoes.—*Culex pipiens* and *Aedes ægypti* were used. The former were either caught in houses or bred in the laboratory from larvæ; the latter were hatched in the laboratory from dried eggs supplied by Dr. Boyd of the Rockefeller Foundation.

Cages.—The mosquito cages consisted of stiff wire frames made in the form of a cube and covered with ordinary cheese cloth. From one side of the cube the cloth extended in the form of a sleeve which served as the only opening to the cage and through which the mosquitoes or larvæ were manipulated.

Technique of Feeding.—Individual mosquitoes were caught in wide test-tubes, 8" x 1". The mouth of each tube was then covered with cheese cloth, which was held in place by a rubber band. Since mosquitoes do not feed readily under these conditions, they were starved for 24 hours prior to the experimental meal. Feeding

was further facilitated by first holding the gauze-covered end of the tube containing the mosquito towards the source of light. The mosquito was attracted by the light and came to rest on the gauze. The tube was then manipulated without disturbing the mosquito so that the gauze-covered mouth of the tube was placed over the area of the comb or wattle to be fed upon. If the mosquito was hungry and its proboscis gently came in contact with the skin of the chicken, feeding promptly ensued. Sometimes a great deal of patience was required. As a rule, however, no great difficulty was encountered in getting the mosquitoes to feed on or near lesions.

The feeding once begun could be interrupted at any time by gently lifting the tube. To prevent infection arising from the apparatus, immediately after the feeding had been interrupted the gauze was removed, the lip of the tube was washed with alcohol and dried, and a clean piece of gauze was placed over the mouth of the tube. At different intervals of time following the infectious meal the mosquito was made to refeed on normal chickens. During the process of refeeding the insect often interrupted its meal either because of an unsatisfactory probe or because the operator moved the tube. If the interruption was brought about so gently that the mosquito remained on the gauze the meal was immediately resumed at a new place. The natural or imposed interruptions proved to be important because in this manner a single mosquito has been shown to be able either to infect a fowl in several places or to infect more than one fowl.

Each tube and each chicken were numbered and records were kept of the time and place of the different feedings. After the completion of each meal the tubes containing the mosquitoes were placed upright in a wire basket and covered with a moist towel. In order to determine the survival time of the virus, the infected mosquitoes were fed on normal chickens at intervals of 3 or 4 days. At various intervals of time following the infecting meal, mosquitoes were also killed, macerated, and injected into the comb or wattles of a fowl.

Eighteen experiments were conducted, 10 with *Culex pipiens* and 8 with *Aedes aegypti*. The results are shown in the following protocols.

Culex pipiens.

Experiment 1.—Sept. 26, *Culex* 2 fed on the infected comb of Chicken 46, drawing whitish fluid. 1 hour later the insect made several insertions of its proboscis, without feeding, on the left side of the comb of Chicken 47. Oct. 5, 4 small vesicles were observed at the points where the mosquito had attempted to refeed. Oct. 8, lesions larger (Fig. 1). Fluid from two of the vesicles was removed by means of a capillary pipette and inoculated on the scarified comb of Chicken 56. 4 days later the comb of Chicken 56 showed definite lesions which progressed and endured for many days; Figs. 4 and 5 are photographs of Chicken 56 taken a week and a month, respectively, after inoculation. Oct. 10, lesions still increasing in size (Fig. 2). Oct. 22, lesions still present (Fig. 3). Oct. 25, lesions regressing.

Experiment 2.—Sept. 26, *Culex* 3 fed on the infected comb of Chicken 46, drawing blood. 45 minutes later the insect refed on the right side of the comb of Chicken 47. No lesions developed at the points of refeeding.

Experiment 3.—Sept. 26, *Culex* 4 fed on the infected wattle of Chicken 45, drawing blood. 45 minutes later the mosquito refed on the right wattle of Chicken 47. No lesions developed at the points of refeeding.

Experiment 4.—Sept. 28, *Culex* 5 fed near lesions on the infected comb of Chicken 46, drawing blood, and 15 minutes later refed, 1 bite, on the left wattle of Chicken 51. Oct. 5, a vesicle appeared on the wattle of Chicken 51 at the point where the mosquito had refed 7 days previously. Oct. 11, lesion larger (Fig. 6). Oct. 22, small secondary nodules were observed on the wattle (Fig. 7). The chicken was tested, Nov. 10, for immunity to fowl-pox virus.

Experiment 5.—Sept. 28, *Culex* 6 fed near lesions on the infected comb of Chicken 46, drawing blood. 15 minutes later the insect refed, 3 bites, on the left comb of Chicken 51. Oct. 5, 2 vesicles were noticed on the comb of Chicken 51, where the mosquito had refed. Oct. 11, lesions increasing in size (Fig. 6). Oct. 22, lesions still increasing in size (Fig. 7). Oct. 26, lesion regressing. Nov. 10, Chicken 51 was reinoculated with fowl-pox virus. Abortive lesions appeared rapidly and were gone within 9 days after the reinoculation.

Experiment 6.—Oct. 15, *Culex* 7 fed on the infected comb of Chicken 57, and 1 hour and 30 minutes later refed, 2 bites, on the left wattle of Chicken 59. Oct. 25, on the left wattle, at the points where the mosquito had refed, 2 vesicles were observed. The lesions progressed and were active for many days (Fig. 8). Nov. 17, definite evidence of healing.

Experiment 7.—Oct. 15, *Culex* 8 fed on the infected comb of Chicken 57, and 1 hour and 30 minutes later refed, 1 bite, on right side of the comb of Chicken 59. Oct. 22, a vesicle was noticed on right side of comb at the point where the insect had refed. The lesion progressed and was active for many days. Nov. 17, definite evidence of healing.

Experiment 8.—Oct. 18, *Culex* 9 fed on the infected comb of Chicken 57. 30 minutes later the insect finished its meal on the right wattle of Chicken 59. Oct. 25, at the points where the mosquito had bitten Chicken 59, 2 lesions, which progressed and evidenced activity for many days, were seen. Nov. 17, definite evidence of healing.

Culex 9 was kept alive for further refedings.

Oct. 22, *Culex* 9 refed on the right wattle of Chicken 63. No lesions developed. This chicken was tested, Nov. 10, and found to be immune to fowl-pox.

Oct. 25, *Culex* 9 refed on the right side of comb of Chicken 66. A questionable lesion appeared, Oct. 29, and regressed in a few days. This chicken was tested, Nov. 10, and found to be immune to fowl-pox.

Oct. 30, *Culex* 9 was macerated and injected into the comb of Chicken 69. No lesion developed. This chicken was tested, Nov. 10, and found to be immune to fowl-pox.

It appears that the refeedings of *Culex* 9 on Oct. 22 and 25 took place on immune fowls. The chicken into which the macerated insect was injected also seems to have been immune.

Experiment 9.—Oct. 18, *Culex* 10 fed on the infected comb of Chicken 57, and 30 minutes later finished its meal on the left side of the comb of Chicken 59. Oct. 25, 2 small lesions were observed on the left side of the comb of Chicken 59. These lesions progressed and remained active for many days (Fig. 8). Nov. 17, definite evidence of healing.

Culex 10 remained alive and was refed on 3 occasions.

Oct. 25, *Culex* 10 refed on the right side of the comb of Chicken 63. No lesions developed. This chicken was tested, Nov. 10, and found to be immune to fowl-pox.

Oct. 31, *Culex* 10 refed on the right side of the comb of Chicken 69. No lesions developed. This chicken was tested, Nov. 10, and found to be immune to fowl-pox.

Nov. 1, *Culex* 10 refed on the anterior part of the right wattle of Chicken 70. After being refed the mosquito was macerated and injected into the right side of the comb of the same fowl. No lesion developed at the point of injection, but on Nov. 5, 2 definite lesions were observed at the points where the insect had refed. These lesions progressed and were photographed, Nov. 7 (Fig. 10). On Nov. 9, 1 of the lesions was excised and fixed in Zenker's fluid. Stained sections showed a typical fowl-pox lesion with Bollinger bodies in the epithelial cells (Fig. 14). Material from the other lesion was inoculated on the scarified comb of Chicken 73. Typical fowl-pox lesions developed.

The lesion on Chicken 70 that was not excised and from which material was removed for transfer began to regress on Nov. 12 and by Nov. 19 was completely healed. The lesion appeared quickly and healed rapidly. It is possible that Chicken 70 was partially immune.

From the experiments recorded above it is evident that *Culex* mosquitoes are able to transmit fowl-pox from infected to susceptible normal chickens. In 7 of 9 tests, infection occurred in the susceptible fowls which had been bitten by mosquitoes that had fed on infected birds 15 minutes to 2 hours previously. In 1 of 2 experiments, *Culex* 10, in which the mosquitoes remained alive for a number of days, a typical infection was produced in a fowl 14 days after the insect had taken its infective meal. The negative results obtained with the other mosquito, *Culex* 9, were probably due to the fact that the chickens on which it refed were immune to fowl-pox.

Aedes ægypti.

Experiments similar to those with *Culex* mosquitoes were conducted with *Aedes*. The results are recorded below.

Experiment 10.—Oct. 22, *Aedes* 1 fed on the infected comb of Chicken 57. 2 hours later the insect completed its meal on the left wattle of Chicken 63. No lesions developed. This chicken was tested, Nov. 10, and found to be immune to fowl-pox.

Oct. 24, *Aedes* 1 refed on the left wattle of Chicken 66. No lesion developed. This chicken was tested, Nov. 10, and found to be partially immune to fowl-pox.

Oct. 30, *Aedes* 1 refed on the left wattle of Chicken 68. On Nov. 5, a lesion appeared where the insect had fed, and subsequently developed into a typical fowl-pox or contagious epithelioma wart.

After *Aedes* 1 had fed, Oct. 30, on Chicken 68, it was macerated and injected into the left side of the comb of Chicken 68. On Nov. 16, a typical fowl-pox lesion was observed at the point where the injection was made.

Experiment 11.—Oct. 22, *Aedes* 2 fed on the infected comb of Chicken 57, and 30 minutes later completed its meal on the left side of the comb of Chicken 63. No lesions developed. This chicken was tested, Nov. 10, and found to be immune to fowl-pox.

Experiment 12.—Oct. 24, *Aedes* 3 fed on the infected comb of Chicken 57. The insect finished its meal 1 hour later on the left side of the comb of Chicken 66. No lesions developed. This chicken was tested, Nov. 10, and found to be partially immune to fowl-pox.

Oct. 26, *Aedes* 3 refed on the left side of comb of Chicken 67. No lesions developed. This chicken was tested, Nov. 10, and found to be immune to fowl-pox.

Oct. 29, *Aedes* 3 refed on the posterior portion of the right side of the comb of Chicken 68. On Nov. 7, 3 vesicles appeared where the mosquito had fed and later developed in typical fowl-pox warts. Nov. 12, photographed (Fig. 9).

Oct. 31, *Aedes* 3 was macerated and injected into the right wattle of Chicken 72. No virus of fowl-pox could be detected in the wattle excised 9 days later.

Experiment 13.—Oct. 24, *Aedes* 4 fed on the infected comb of Chicken 57, and 1 hour later completed its meal on the left side of the comb of Chicken 66. No lesions developed. This chicken was tested, Nov. 10, and found to be partially immune to fowl-pox.

Oct. 26, *Aedes* 4 refed on the right side of the comb of Chicken 67. No lesions developed and the chicken was later found to be immune.

Experiment 14.—Oct. 25, *Aedes* 5 fed on the infected wattle of Chicken 51, and a few minutes later finished its meal on the right side of the comb of Chicken 66. No lesions developed and the chicken subsequently was found to be immune to fowl-pox.

Experiment 15.—Oct. 26, *Aedes* 6 began its meal on the infected wattle of Chicken 61 and finished it, 2 bites, on the right wattle of Chicken 67. A doubtful lesion appeared, Nov. 3, and disappeared within a few days. Chicken 67 was tested, Nov. 10, and found to be immune to fowl-pox.

Oct. 28, *Aedes* 6 refed in the center of the right side of the comb of Chicken 68.

No lesions appeared. Chicken 68 was definitely susceptible (see Experiments 10 and 17).

Oct. 31, *Aedes* 6 refed on the left wattle of Chicken 69. No lesions developed and the fowl was later found to be immune.

Nov. 3, *Aedes* 6, with *Aedes* 8, was macerated and injected into the right side of the comb of Chicken 72. No lesions developed.

Experiment 16.—Oct. 26, *Aedes* 7 fed on the infected wattle of Chicken 61, and a few minutes later finished its meal on the left wattle of Chicken 67. On Nov. 1, a small lesion appeared which persisted for only 3 days. Chicken 67 was tested, Nov. 10, and found to be immune to fowl-pox.

Oct. 29, *Aedes* 7 refed on upper part of the right wattle of Chicken 68. No lesion developed. The chicken was definitely susceptible (see Experiments 10 and 17).

Oct. 31, *Aedes* 7 refed on the central portion of the right side of the comb of Chicken 69. No lesion developed. The fowl was subsequently shown to be immune to fowl-pox.

Nov. 3, *Aedes* 7 was macerated and injected into the left wattle of Chicken 72. No fowl-pox virus was demonstrated in the wattle excised 9 days later.

[*Experiment 17.*—Oct. 26, *Aedes* 8 fed on the infected wattle of Chicken 61. A few minutes after the meal was interrupted it was completed on the upper part of the left wattle of Chicken 67. No lesion appeared. Chicken 67 was shown subsequently to be immune.

Oct. 29, *Aedes* 8 refed on the lower part of the right wattle of Chicken 68. On Nov. 3, 2 small nodules were observed at the points where the insect had refed. Nov. 12, lesions photographed (Fig. 9). These nodules developed into typical fowl-pox lesions.

Nov. 1, *Aedes* 8 refed on the posterior part of the right wattle of Chicken 70. No lesion developed. This chicken was certainly partially susceptible at the time it was bitten (see Experiment 9).

Nov. 3, *Aedes* 8, with *Aedes* 6, was macerated and injected into the right side of the comb of Chicken 72. No lesion developed.

The results of the experiments with *Aedes* mosquitoes were not as uniformly positive as were those with the *Culex*. Eight *Aedes* mosquitoes were fed on infected chickens and later, at different intervals of time following the infective meal, were refed on healthy fowls. Of the 8 refeedings that were made within 2 hours of the infective meal only 2, *Aedes* 6 and 7, resulted in lesions at the points where the insects bit. These lesions were small and disappeared within a few days. Refeedings of *Aedes* 1, 3, and 8, which took place 9, 5, and 3 days respectively after the primary feeding, gave rise to typical fowl-pox lesions. The many negative results obtained in these experiments

were probably due to the fact that 4 of the 6 chickens, 63, 66, 67 and 69, employed were subsequently found to be either partially or completely immune to fowl-pox. Sufficient positive results were obtained, however, to show that *Aedes* mosquitoes are capable of inoculating healthy susceptible chickens with the virus of fowl-pox many days after they have fed on infected combs of diseased birds.

Transmission of Fowl-Pox by Mosquitoes under Natural Conditions.

The results of the work reported above show that *Culex* and *Aedes* mosquitoes are capable of transmitting fowl-pox. The tests, however, were made under experimental, not natural, conditions. It seemed advisable, therefore, to conduct at least 1 experiment in which the conditions approximated those occurring in nature.

Experiment 18.—Oct. 15, 2 healthy chickens (Nos. 61 and 62) and 1 (No. 57) with fresh fowl-pox lesions on both sides of the comb and on both wattles were placed in a mosquito-proof cage (Fig. 12). Five recently hatched *Culex* mosquitoes were also introduced into the cage. Oct. 22, 7 days after the experiment was started, 2 small lesions were observed on the lower part of the left wattle of Chicken 61. Oct. 26, lesions on Chicken 61 were still increasing in size (Fig. 11). One of the lesions was removed and fixed in Zenker's fluid. Stained sections (Fig. 13) showed a typical early fowl-pox lesion with many Bollinger bodies in the injured epithelial cells. The other lesion which was not excised went through the evolution usually observed in fowl-pox.

Chicken 62 never developed any lesions. This may have been due to the fact that it was immune to fowl-pox before the experiment was begun. In any event, it was tested, Nov. 10, and found to be immune at that time. It is unfortunate that tests for immunity cannot be made before the fowls are used for experimental purposes. Chicken 61, that showed the 2 typical lesions, later developed an immunity, which was probably acquired through the infection contracted during the experiment.

The control for the experiment was conducted in a manner similar to that employed in the test, with the exception that mosquitoes were excluded from the cage. 2 healthy chickens (Nos. 77 and 79) were placed in a cage with a fowl (No. 73) that had fresh fowl-pox lesions on the comb. The healthy chickens frequently pecked at the warty growths on the comb of the infected fowl. In spite of this close contact with the infectious agent, the healthy birds developed no lesions during the 12-day period of observation. They were later shown to be susceptible to fowl-pox.

The results of Experiment 18 clearly indicate that mosquitoes, under natural conditions, can transmit fowl-pox from diseased to healthy susceptible chickens.

Nature of the Lesions Produced by Insect Inoculation.

To present a study of the pathology of fowl-pox is not the purpose of this paper. It seems desirable, however, to describe the lesions produced by insect inoculation.

At the points on the comb or wattles of susceptible chickens where infected mosquitoes had fed or attempted to feed, minute nodules usually appeared within 5 to 9 days. Such lesions rapidly increased in size and assumed a grayish, glistening, translucent appearance. Frequently within 2 or 3 days the nodules were partially transformed into vesicles from which, by means of a capillary pipette, small amounts of whitish fluid rich in virus could be obtained (Figs. 1, 10, 11). If undisturbed the lesions continued to increase in size and gradually became large, yellowish, warty growths (Figs. 3 and 7). Finally regression and healing supervened and the wart-like scabs fell off, leaving superficial white scars. In non-immune fowls with 2 to 8 lesions, the duration of the disease was 3 to 6 weeks. In what appeared to be partially immune chickens, however, the incubation period was short, only 4 days at times, and the evolution of the disease was rapid, frequently requiring less than 2 weeks (see Experiment 9).

In certain instances, the stained sections of young lesions presented a marked hyperplasia of the epithelial cells with little, if any, reaction in the corium (Fig. 13a). In the case of 1 fowl, however, a lesion, approximately of the same age as the ones above described showed, in addition to the hyperplasia, vesicles in the epithelial layer and a marked cellular reaction in the corium (Fig. 14a). An explanation of this difference in the amount of reaction in the corium is not possible at present. Possibly this difference can be accounted for upon the ground that the depth of the insertion of the insects' proboscis varied considerably with each feeding and attempted feeding or that the reactivity of the fowls, without respect to specific resistance or immunity, was not the same in every case. Our impression, however, is that the marked early reaction in the corium occurred in a partially immune fowl (Experiment 9). This idea is at least in agreement with some observations recently reported by Andrewes (11) in regard to the histology of Virus III lesions in partially immune rabbits. From our studies it is clear that in some early fowl-pox lesions produced by the bites of infected mosquitoes a marked involvement of the epithelial cells may occur before any definite evidence of a reaction is discernible in the corium.

DISCUSSION.

To incriminate an insect as an important vector of disease, it is essential to establish the fact that it bites readily and comes in close contact with the susceptible hosts. The intimate relation between fowls and mosquitoes has long been recognized. Furthermore, *Culex* mosquitoes feed readily on birds and are known to transmit bird malaria.

Under the conditions of the experiments reported in the present paper it is evident that *Culex* and *Aedes* mosquitoes are capable of transmitting fowl-pox from diseased to healthy susceptible chickens. Moreover, the indications are that these insects may play an important rôle in the rapid and thorough infestations of flocks under natural conditions. The question, however, as to whether this mode of transmission is the most significant one remains to be answered by experiments conducted in the field. Certainly there is no reason why other biting insects may not also be of importance (10).

While in most epidemics of fowl-pox the lesions occur on the chickens' comb and wattles, in occasional outbreaks the majority of the manifestations of the disease is found in the mouth and throat. Inasmuch as the mucous membranes are inaccessible to mosquitoes and since the eating of virus alone usually does not result in infection, the spread of this type of the disease is hard to relate either to mosquitoes or to the simple ingestion of virus. It is possible that another infection, e.g., bacterial, may injure the mucous membranes, thus making them susceptible to fowl-pox. In this manner a combination of infectious agents may account for the unusual and frequently fatal form of the disease.

It has been shown that mosquitoes are capable of transmitting fowl-pox at various times during the first 14 days following the infective meal. Tests at intervals longer than 2 weeks were not made. Therefore, the total duration of infectiousness of the insects remains to be determined. In view of the fact that the virus is active in minute quantities and is highly resistant to drying, the results of our experiments can be explained entirely upon the grounds that the mosquitoes mechanically transmit the disease without the occurrence of any multiplication of the virus in the insects. Before definite conclusions

can be reached, however, this phase of the problem will require further investigation.

The fact that insects may play a definite part in the rapid spread of certain diseases by mechanically transferring the virus from diseased to healthy individuals has been recognized by other investigators (13-16). This applies particularly to the virus diseases of plants (17). Nevertheless, from the results of our experiments, it appears that the importance of this mode of dissemination of certain virus diseases (12) of animals has either been underestimated or not sufficiently investigated.

CONCLUSIONS.

Culex and *Aedes* mosquitoes are capable of transmitting fowl-pox from diseased to healthy susceptible chickens.

The mosquitoes remain infectious for at least 14 days following a meal on diseased fowls.

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EXPLANATION OF PLATES.

PLATE 38.

FIGS. 1 to 3. Photographs of Chicken 47, showing the development of 4 fowl-pox lesions caused by the bites of an infected *Culex* mosquito.

FIGS. 4 and 5. Photographs of Chicken 56 that was inoculated with infectious material taken from the lesions on Chicken 47.

PLATE 39.

FIGS. 6 and 7. Photographs of Chicken 51, showing the development of fowl-pox lesions induced by the bites of infected *Culex* mosquitoes. *a* is primary lesion; *b* are secondary nodules.

FIG. 8. Chicken 59 with lesions produced by the bites of infected *Culex* mosquitoes.

FIG. 9. Chicken 68 with lesions induced by the bites of infected *Aedes* mosquitoes.

FIG. 10. Chicken 70 with 2 young fowl-pox lesions on the wattle induced by the bite of *Culex* 10, 14 days after its infectious meal. Lesion *a* was removed for histological study (see Fig. 14).

FIG. 11. Chicken 61 with 2 young fowl-pox lesions on the wattle. The bird contracted the disease in a cage in which healthy and infected fowls were placed with *Culex* mosquitoes. Lesion *a* was removed for histological study (see Fig. 13).

PLATE 40.

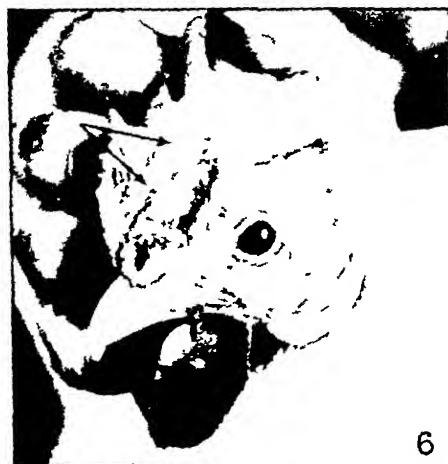
FIG. 12. The cage in which the transmission of fowl-pox by mosquitoes under natural conditions was tested.

PLATE 41.

FIG. 13. *a* represents a section of a young fowl-pox lesion taken from Chicken 61. Note the hyperplasia of the epithelial cells and the absence of reaction in the corium. $\times 50$. *b* represents a normal epithelial cell. $\times 1200$. *c*, *d*, *e* represent epithelial cells with Bollinger bodies in their cytoplasm. $\times 1200$.

FIG. 14. *a* represents a section of a young fowl-pox lesion removed from Chicken 70. Note the hyperplasia of epithelial cells, the vesicles in the epidermis, and the marked reaction in the corium. $\times 50$. *b*, *c*, *d*, *e* represent epithelial cells with Bollinger bodies in their cytoplasm. $\times 1200$.





6



7



8



9



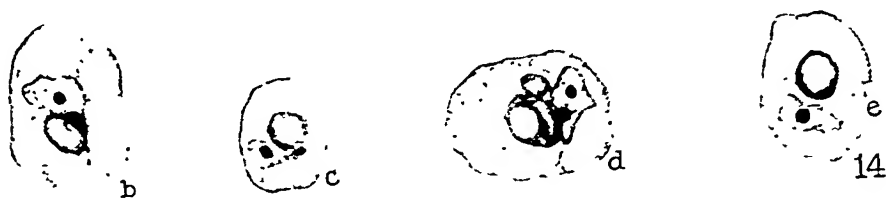
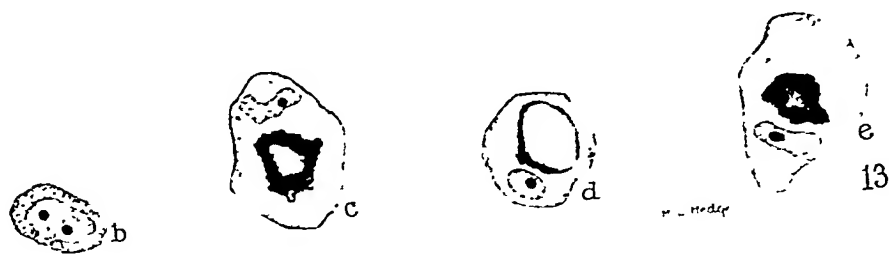
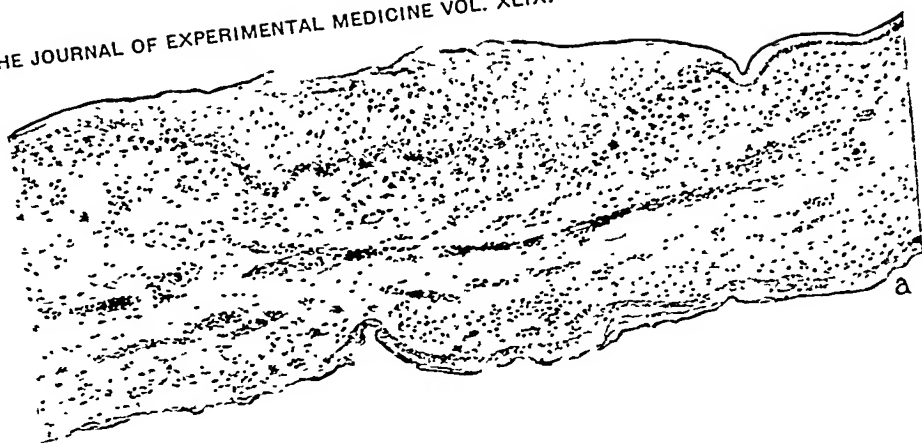
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11



(Kligler, Muckenfuss, and Rivers: Fowl-pox.)



HERPES ENCEPHALITIS IN CEBUS MONKEYS.

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PLATES 42 AND 43.

(Received for publication, December 11, 1928.)

For a considerable number of years we have been attempting to produce herpetic encephalitis in monkeys, at the same time that we were trying to transfer encephalitis with human material to these animals and rabbits. In spite of the occasional reports in the literature that *Macacus rhesus* and other species of *Macacus* were susceptible to herpes virus, we were entirely unsuccessful in producing any sort of a disease in the *Macacus* species, even with the most potent herpetic strain in our possession. We administered the virus intracerebrally, intraperitoneally, and implanted it in large amounts, together with agar, under the skin. Never did we obtain any reaction in these monkeys. During the past winter, having in our possession a ringtail monkey of the South American *Cebus olivaceus* species, we inoculated this animal with herpes intracerebrally, and found that a disease not unlike that occurring in rabbits killed this animal in the course of 9 days. Since that time we have attempted to produce encephalitis in *Cebus olivaceus* monkeys with the spinal fluid of several cases diagnosed as probable encephalitis, but apart from a curious edema of the face and temporary unwillingness to eat, in one animal, coming on about a week to 3 weeks after inoculation and from which complete recovery occurred, we obtained no results whatever.

Although a *Cebus* monkey inoculated with the spinal fluid from a case at the Boston Psychopathic Hospital did not develop any symptoms which could be regarded as simulating a true encephalitis, the subsequent inoculations of other monkeys carried out with the brain of this one developed into a series of observations which we believe sufficiently interesting and important to record in detail.

Fresh brain from the original spinal fluid monkey, which we shall call *Cebus I*, was inoculated intracerebrally¹ on May 21 into *Cebus II* and into two rabbits. The rabbits remained entirely well, not even developing a temperature. *Cebus II* likewise showed no signs of illness, except that 7 days after inoculation it developed a swollen edematous area on the right side of the face during a period of about a week. The edema then subsided and the monkey returned to normal.

On July 9, material from the first monkey, *Cebus I*, which had now been in glycerol for 21 days, was inoculated into a third monkey, *Cebus III*, and into two rabbits. These two rabbits developed—4 days after inoculation—temperatures rising to 105.1° in one case and 105.8° in the other, but recovered. Transfers to other rabbits from these animals developed no virus effects, and the monkey in this series developed no symptoms whatever.

On August 4, another monkey, *Cebus IV*, was inoculated, again with material from *Cebus I*, now 45 days in glycerol in the ice chest, and this animal also developed a facial edema with a few days of apparent illness evidenced by lassitude and loss of appetite. This monkey completely recovered within a week, and thereafter remained well.

At first we thought that the facial edema which occurred in three separate animals might have some special significance, but concluded that it probably was due to some injury not anatomically traceable, consequent upon the intracerebral inoculations. *Cebus III*, which was killed and examined, had developed upon the edematous area a small staphylococcus abscess. We therefore paid no particular attention to the edema, which we could not then and cannot now explain satisfactorily, and preserved these monkeys for subsequent inoculation with herpes virus, in order to find out whether they had developed any immunity.

The above preliminary history of these monkeys is given for the purpose of exactitude, although we are inclined to believe at the present time that the previous inoculations had little to do with the subsequent histories of the animals. This will appear below.

On October 2, *Cebus IV*, above described, now entirely well, was intracerebrally inoculated with brain material taken from Rabbit 42 and glycerolated for 1 week. This rabbit had died typically after inoculation with an herpetic filtrate. At the same time, a normal *Cebus*, *Cebus V*, was inoculated as a control. The difference between these two monkeys was a striking one, both clinically and pathologically.

Cebus V died as other normal monkeys of this species have died after inoculation with herpes, after an acute illness of 9 days. *Cebus IV*, however, did not show any signs of illness until the 10th day after inoculation. On October 12 it developed

¹ Ether anesthesia was used in all operative experiments.

an unwillingness to eat, tremors and lassitude. This on subsequent days developed into a state of somnolence which by October 15 and 16 was so marked that the monkey squatted in the corner of the cage, rarely changing its position, swaying to and fro with its eyes closed and obviously asleep. The only movements were occasional twitchings of the arms and legs. This condition continued day after day. When aroused and offered food, the monkey would eat and occasionally would make uncoordinated and purposeless waving movements with the hands, alternating with tremors. Immediately upon being set back into the cage, it relapsed into deep somnolence from which it rarely roused itself spontaneously. When the limbs were extended, they went into a temporary spastic condition. The



TEXT-FIG. 1. Habitual position held by *Cebus* IV while somnolent during a period beginning 12 days after inoculation and lasting until killed, 24 days after inoculation.²

monkey was examined during this period by Dr. Stanley Cobb and in its general clinical condition displayed a typical encephalitis of an acute type. On the 24th day after inoculation, the animal seemed to be a little more lively, and in order that we might obtain material for the study of the pathological condition as it then existed, the monkey was etherized and the nervous system removed.

Inoculations were made from the brain of *Cebus* V, which died acutely, into another monkey, *Cebus* VI, and into two rabbits—all of

² All the photographs were taken by Dr. S. Burt Wolbach, to whom I wish to express my appreciation and thanks.

which died acutely and with typical signs of herpes, proving that the condition of which the control monkey had died was one of herpes.

Material from *Cebus* IV, the one with prolonged somnolence, was inoculated into another monkey, *Cebus* VIII, on November 2, and this monkey has shown nothing to date. We neglected at this time to inoculate rabbits from this monkey, but when—by November 15—the virus of *Cebus* IV had failed to kill a monkey of the same species, while the virus of its acutely dead control *Cebus* V, had killed promptly, we inoculated two rabbits with the material of *Cebus* IV, since it seemed that during the chronic condition in this monkey the virus had become either attenuated or destroyed. The material by this time had been in glycerol since October 25, and of course it was an experimental error not to have inoculated rabbits immediately, with the fresh material. This rabbit died of typical, acute herpes. The problem involved will be discussed below. Text-fig. 1 is a photograph of *Cebus* IV, which remained somnolent, in the position which it held practically without change while asleep for 12 days.

The most interesting part of our experiments—indeed, the findings which encouraged us to publish them—are the pathological examinations of the brains. Although all three monkeys of these experiments had the same virus, *Cebus* IV was killed after a prolonged somnolence, whereas V and VI died acutely. The differences in the length of time for which these monkeys survived seem at the same time to have involved important differences of the pathological picture.

The following pathological report is one which was very kindly prepared for us by our colleague, Dr. Wolbach, whose assistance we solicited in order to be sure that there would be no mistake in the pathological interpretation.

Cebus IV.—(This is the monkey which showed no symptoms whatever for 12 days, on the 12th day developed the picture of encephalitis described above and was killed 12 days after the onset—that is, 24 days after inoculation.)

Eight sections of the spinal cord representing different levels are negative. Three sections of the cerebellum show no lesions. Two sections through the basal ganglion, presumably the caudate nucleus and underlying internal capsule, and two sections through the pons and medulla show very numerous and fairly striking perivascular infiltrations, always about blood vessels of medium size. The cells consist chiefly of lymphoid and plasma cells, but there are many cells that have the characteristics of mononuclear phagocytes, rarely, however, containing

inclusions. There are also cells that seem to be of connective tissue origin. This is borne out by the fact that there is undoubtedly an occasional minute cicatrix that has formed in the adventitia, rarely an eosinophil cell. In the immediately adjacent zone of nerve tissue there has been an increase of neuroglia and in the pons and basal ganglion remote from blood vessels of any size are small collections of neuroglia, resembling very much the late typhus lesion and in some instances the glia stars of Spielmeyer. These perivascular infiltrations are most abundant and most striking beneath the ependyma of the lateral ventricles in the basal ganglion and beneath the ependyma of the fourth ventricle. The minute glia cicatrices are found chiefly in the nuclei of the pons and in the basal ganglia on both sides of the internal capsule, and therefore presumably both in caudate and lenticular nuclei. The cerebral cortex is represented by five sections, all of which show lesions. In one section there is very striking diminution of ganglion cells, and it is possible to determine the former location of ganglion cells by clusters of neuroglia. The more acute lesions are represented by clusters of cells, the satellite cells, but throughout there is a very pronounced increase of astrocytes. In this region the perivascular infiltration is very striking. The vessels are surrounded by broad zones of cells composed of lymphoid, plasma cells and an occasional macrophage, a rare eosinophil and fibroblasts arising evidently from the adventitia of the vessel. The pia and arachnoid over this region are heavily infiltrated and here it is possible to see that there has been a very considerable increase of connective tissue, but the picture is again dominated by lymphoid, plasma cells and macrophages. There are a few degenerative ganglion cells in this region with greatly swollen nuclei filled with a finely granular, deeply eosin-staining material. These resemble very closely the inclusions described by Lipschütz (1) and, recently, by Goodpasture (2). The inclusions are typical of herpetic lesions in rabbits. They were described by Goodpasture in ganglion cells and in other tissues. There is a very marked increase of neuroglia cells of the astrocyte type beneath the glia, and everywhere throughout the cortex there is an increase in cell richness. Numerous cells contain large vacuoles and agree in appearances with the oligodendroglia cells which seem on the whole to be increased in numbers. To summarize, the brain of this monkey shows in basal ganglion, pons, medulla and cerebral cortex many perivascular infiltrations. In the gray matter of all these locations there are numerous minute neuroglia cicatrices. In the cortex there are more recent lesions, as evidenced by the finding of degenerated ganglion cells, and neuronophagia. The degree of perivascular infiltration and infiltration of the leptomeninges corresponds to lesions found in human cerebral trypanosomiasis. There are no vascular thromboses. Careful search fails to reveal any recognizable parasites or microorganisms of any sort.

Cebus V.—(This is the monkey which died of acute encephalitis 8 days after inoculation.)

There are sections from five different portions of the cortex and underlying white matter. Sections representing three different regions show a widespread

involvement of the cortex evidenced by lesions of individual ganglion cells, increase in satellite cells and an early perivascular reaction. None of these changes are conspicuous as observed through the low power. The outstanding feature to be observed with the low power is the swelling of nuclei and the presence of nuclear inclusions. Many of the ganglion cells containing these inclusions show little change. Others show very marked degenerative changes. The inclusions are those typical of the herpetic infection as described by Goodpasture, Lipschütz and many other authors. The earliest form seems to be a deeply staining, irregular, somewhat ameboid appearing inclusion. In every instance there is disappearance of the nucleolus. Later stages are represented by nuclei very markedly distended, outlined by a deeply staining, nuclear membrane, presumably containing peripherally displaced chromatin bodies. The inclusion itself in these conditions seems to consist of a mass of finely granular, neutral staining material. With the highest powers and best illumination, one gets the impression that it is composed of minute granules, tightly packed together. These degenerative ganglion cells containing these nuclear inclusions are always accompanied by several greatly enlarged satellite cells, and neuronophagia is in progress. Here and there throughout the cortex there are accumulations of cells in and about the adventitia of the blood vessels. These cells are of an indiscriminate type for the greater part and resemble macrophages or are young fibroblasts. Only occasionally can cells be identified as belonging to the lymphoid series. The appearances on the whole are those that give support to the idea that the lymphoid and plasma cells in perivascular infiltration arise from indifferent cells which take origin in the adventitia of blood vessels. There are a few foci where there is a much greater reaction, where ganglion cells are completely necrotic and surrounded by clusters of enlarged phagocytic cells. Around capillaries there are occasional polymorphonuclear leucocytes. In these areas and in less focal distributions there is a much more pronounced perivascular infiltration. Every ganglion cell is involved, —that is, containing nuclear inclusion with degeneration of the cell body. There are numerous polymorphonuclear leucocytes adjacent to the cells and migrating through the brain itself, and also in the zone of perivascular infiltration. Rarely there is an eosinophil about a blood vessel. The meninges show very little change. There is a slight infiltration with cells that appear to be of the lymphocytic series, although larger than the cells in *Cebus* IV (Fig. 3). Here again, are undifferentiated cells in the pia which could be taken either for fibroblasts or macrophages and suggest origin *in situ* of the lymphoid and plasma cell so commonly found in encephalitis of diverse causation. Without going into great detail, one can characterize this brain by saying that there is an extraordinarily widespread or diffuse involvement of the ganglion cells resulting in their degeneration, the formation of nuclear inclusion and the usual response on the part of the satellite cells. The acuteness of the process is evidenced by the appearance of polymorphonuclear leucocytes and the early reaction around blood vessels and in the meninges. If I should venture a guess, I should express the opinion that this slide indicates that the initial damage

to the brain has been in the ganglion cell of the cortex. In other words, that there has been a specific localization of the injurious agent in the cell. The other changes can be interpreted as secondary to the destruction of the ganglion cells. The suggestion by Lipschütz that these inclusions are due to the actual presence of the virus is not difficult to believe after seeing these preparations.

Figs. 1 and 2 represent the typical, perivascular infiltration in the brain of the monkey with the more slowly progressing condition, simulating human encephalitis. Fig. 4 is a high power drawing of the swollen, degenerated nuclei and nuclear inclusions found in such large numbers in the monkey which died in 8 days.

DISCUSSION.

The observations which we have outlined reveal that *Cebus olivaceus* monkeys inoculated with the identical herpetic virus may react in two quite different ways, both clinically and pathologically, according to the acuteness with which the disease progresses. In the monkey which died acutely in 9 days, we have a clinical picture which has no definite similarity to human encephalitis, but resembles in its clinical and pathological features the condition observed in rabbits after inoculation with herpes virus. The lesions in this acute animal involve the ganglion cells and changes in the nuclei with the nuclear inclusions described by Lipschütz and by Goodpasture and Teague (3) in the brains of rabbits. The perivascular infiltrations observed in the typical encephalitic disease of man are relatively undeveloped and of a minor type.

In the more chronic form of the disease, on the other hand, in which we had a clinical picture strikingly like that of encephalitis in man, the pathology as well is one more closely resembling that of man, and although there are a few areas with nuclear inclusions and changes similar to those observed in the acute animal, the perivascular infiltrations and other changes are remarkably like those of human encephalitis.

It would appear from this that the same virus, when not acutely fatal, produces lesions quite different from those in the acute cases; the differences in picture being to some extent, therefore, dependent upon the length of time and the violence with which the agent is active in the central nervous system. In following the sequence of our experiments, the thought is obvious that perhaps *Cebus IV*, the animal which was more chronically ill and might have recovered, was partially

immunized by a preceding inoculation some months before with the brain of a monkey into which material from a case of human encephalitis had been injected. This would be significant etiologically, were it maintainable; but we are inclined to believe that the difference between this monkey and several others that have died acutely is one of variation of susceptibility in the individual animal, which becomes manifest when a virus of somewhat reduced potency is used.

Of considerable interest, also, is the fact that a *Cebus* monkey and two rabbits inoculated with the brain of the acute monkey died typically of acute herpetic encephalitis, whereas a similar monkey inoculated with the brain of the chronically diseased animal has shown no symptoms to date. This observation would indicate that there was some attenuation of the virus in the case of the more prolonged pathological reaction in this animal, a condition which has been referred to as autosterilization by Levaditi, and might explain the almost universal failure of transmission to animals with materials from human encephalitic cases. It must, however, be mentioned that subsequent inoculations of rabbits with the brain of this chronic monkey produced typical herpes encephalitis.

Our experiments have shown that, given the proper degree of balance between infectiousness and resistance in a susceptible species of monkey, herpes virus may give rise either to an acute disease resembling clinically and pathologically the acute herpetic encephalitis of rabbits, or to a more prolonged malady in which both clinical features and pathological lesions are strikingly similar to the human disease.

We have no idea that this solves the problem of the etiology of epidemic encephalitis, in the sense of an acceptance of the herpes virus as an etiological factor. It merely shows that the typical clinical and pathological pictures are as closely dependent upon acuteness or chronicity of the disease as they are upon the nature of the virus. Indeed, in the years during which we have unsuccessfully attempted to transfer human encephalitis to rabbits and monkeys, we have had occasion to obtain material from spontaneous encephalitis, from encephalitis following vaccination and from a recent case in which an apparently typical encephalitis followed treatment with phenolized rabies virus. The literature cites encephalitis as most frequently following influenza, vaccination, measles, varicella—all of them, except influenza, so

called "filtrable virus diseases," and influenza still uncertain in its etiology.

It would seem to us most rational on the basis of our own experience and the general experience of others to assume for the present that the clinical and pathological injuries grouped together as encephalitis might be due to the development of neurotropism by a number of different filtrable agents, and that the similarity in pathology is due to an analogous reaction on the part of the tissues to various members of a single group of closely related agents. The fact that Rivers and Stewart (4) have recently been able to produce encephalitis with Virus III would seem to further encourage the idea that encephalitic disease represents a clinical group in which herpes virus may be occasionally responsible, but in which the neurotropism of a considerable number of other filtrable agents is involved.

CONCLUSIONS.

Herpes virus which ordinarily produces in *Cebus olivaceus* monkeys an acutely fatal encephalitis closely resembling in time, symptoms and pathology the acute, herpetic disease of rabbits may—in more resistant individual monkeys—lead to a more prolonged malady which, while unquestionably produced with herpes virus, simulates with considerable accuracy the human disease of acute encephalitis, in symptoms, in course and in pathological changes.

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EXPLANATION OF PLATES.

PLATE 42.

Sections were stained both by the ordinary hematoxylin-eosin and by the Giemsa method. The latter was the method that showed cellular details most satisfactorily.

FIG. 1. Section through brain of *Cebus* IV, monkey with typical encephalitis, showing perivascular infiltration.

FIG. 2. Section through the same brain as Fig. 1.

PLATE 43.

FIG. 3. Brain of a monkey with acute encephalitis. To show the condition of the nuclei and inclusion bodies.

FIG. 4. Oil immersion drawings of nuclear swellings and inclusions in the ganglion cells of the brain of *Cebus* V, the control on *Cebus* IV, but which—in contrast to *Cebus* IV—died acutely.

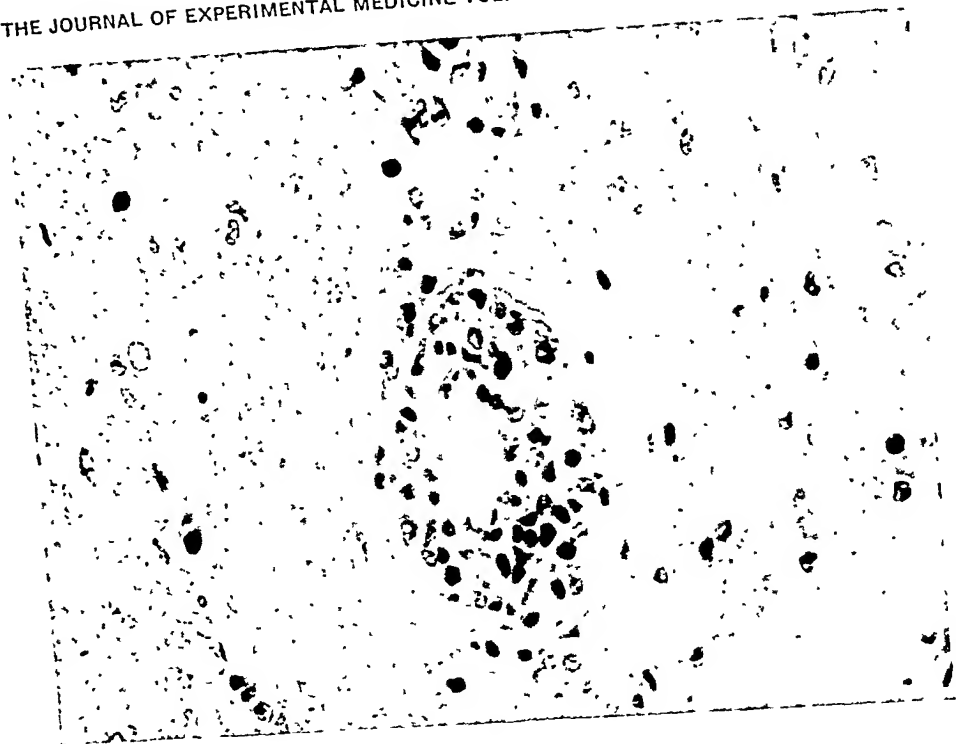


FIG. 1.

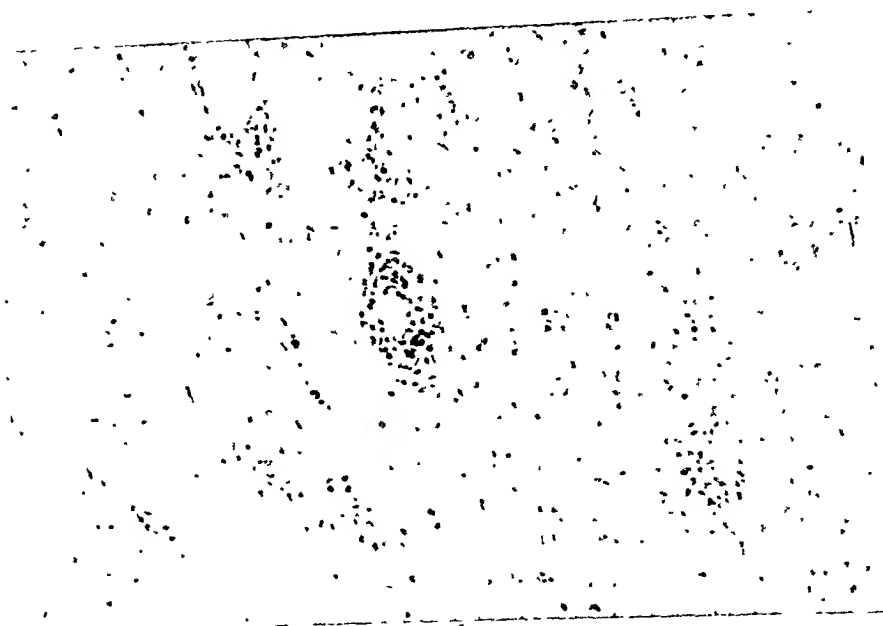


FIG. 2

(Zinsser: Herpes encephalitis in *Cebus* monkeys.)



FIG. 3.

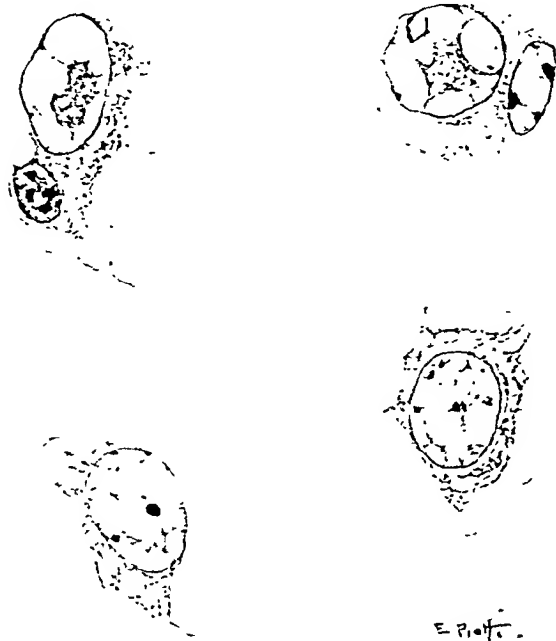


FIG. 4.

(Zinsser: Herpes encephalitis in *Cebus* monkeys.)

A STRAIN OF BACILLUS ABORTUS FROM SWINE.

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The economic significance of abortion among swine and the evidence already presented that the particular race or races of *Bacillus abortus* causing this disease may be involved in undulant fever of man¹ justify a rather detailed study of this disease and the strains of *Bacillus abortus* associated with it.

A review of the early work with the porcine disease was published in 1926.² The evidence up to that date, though quite incomplete, pointed to an identity with the bovine races in terms of specific agglutinins but to differences in growth in sealed and unsealed cultures and in the lesions produced in guinea pigs. In these animals suppuration following the characteristic proliferation of monocytes into tubercle-like foci in this whole group was stressed as an important feature of the porcine races of *Bacillus abortus*. At that time no original strains were obtainable.

Late in May, 1927, our attention was called to an outbreak of swine abortion in the herd of a State institution.³ With the effective co-operation of the State authorities we were able to obtain a few fetuses. At the same time it was learned that the animals had not been fed cow's milk and that the garbage from the institution was not heated before it was fed. The yards were infested with rats. There had been no record of abortion in this herd prior to March of this year. When it occurred the aborted fetuses and membranes appeared decomposed to the attendants. A white viscid discharge appeared around the vulva of the affected sows. There had been no fresh pigs introduced

¹ Smith, T., *J. Exp. Med.*, 1926, xliii, 207.

² Smith, T., *J. Exp. Med.*, 1926, xliii, 215.

³ The writer is indebted to Dr. R. B. Little of this Department for data and material from this outbreak.

into the herd except a boar in 1926 which came from a herd free from the disease. Since the porcine disease has been subjected to but little detailed study, perhaps owing to its relatively infrequent occurrence, the notes on the several cases are briefly reproduced here.

No. 401.—Fetus of sow, reported to have been born dead and buried soon after. The matter was of sufficient importance at this time to warrant the recovery and study of this fetus, which had been buried overnight. Autopsy indicates that fetus had breathed. Several pieces of straw from bedding had penetrated into thorax and abdomen. Stomach empty, inflated with gas. Organs not noticeably affected. The placenta obtained in part shows irregular thin white patches of rather tough consistency, 1 to 2 cm. in diameter, which are readily peeled off the chorion. They resemble fat. Under the microscope the material composing them is amorphous and yields gas when 2 per cent HCl is passed under cover-slip. No exudates or ulcers noticed.

Owing to the condition of the fetal carcass only cultures from heart's blood and liver were made. In both blood cultures about 25 colonies appeared within 3 days. Colonies vary from $\frac{1}{2}$ to $1\frac{1}{2}$ mm. in diameter, and are convex, grayish, glistening. In the liver culture the same type of colony appeared. These were later identified as *B. abortus*.

Sow 402.—Discharged two fetuses (402a and 402b) June 2, 1927. Duration of pregnancy 7 to 8 weeks. *Fetus 402a*, 23 cm. long, from tip of snout to root of tail. Slight effusion of clear serum in serous cavities and pericardial sac. Heart pale, almost white. Liver and kidneys full of blood. Stomach contains a glass-clear thick fluid, resembling aqueous humor. In it many fecal pellets. Lungs not inflated. Only a small piece of placenta obtained. On it minute grayish deposits. A few cocci seen in films from it.

Fetus 402b, identical as to size and condition with 402a. About one-half of placenta obtained. The surface of the chorion is overlaid with very thin isolated grayish patches, 2 to 3 mm. in diameter. A few lumps of exudate lying on surface. Films of scrapings show masses of cells, chiefly polymorphs, many containing three or more bacilli a trifle larger than the bovine type of *B. abortus*. Some are spindle-shaped. The subchorionic tissue is slightly edematous.

Cultures from these fetuses gave the following result:

402a.—Cultures of spleen bit and stomach fluid sterile; of heart's blood contain a streptococcus; of lung and liver, rich growths resembling *B. coli*.

402b.—Cultures of stomach contents and liver remain sterile; of spleen contain a large coccus. A tube inoculated with heart's blood has a heavy growth above the condensation water; on the slope two small colonies later identified as *B. abortus*. Subcultures from these colonies develop in 24 hours without seal. The growth is moderately heavy in 48 hours.

Sections of hardened tissues from various organs of Fetuses 402a and b did not bring out any recognizable lesions. Similarly sections of the placentas were

negative. In view of the definite lesions found in the fresh material, the sections probably did not pass through any diseased areas.

Sow 403.—Farrowed a litter of 9 small living pigs. On the assumption that the uterus might contain *B. abortus*, a swab was introduced into the vagina to collect a whitish viscid discharge which had appeared. This was stirred in bouillon and two guinea pigs inoculated subcutaneously with the clouded suspension. When killed 29 days later both animals had gained considerably in weight. The autopsy and spleen cultures were negative in both cases.

Sow 404.—Farrowed a litter of 5 pigs of which 2 were born dead. These were buried together with parts of placenta. Next day, the fetuses were dug up and a uterine swab obtained from the sow.

Fetus 404a.—Male, 35 cm. long. Abdomen contains fluid blood. Lungs not inflated. No abnormalities noted. In making cultures bits of tissue and several drops of stomach fluid were transferred. Pure cultures of *B. abortus* were obtained from liver, heart's blood, lungs, and stomach fluid. Only a few colonies developed in spite of the large inoculum. All are convex, almost hemispherical droplets, grayish, glistening, partly translucent, measuring 1.5 to 2 mm. in diameter.

Fetus 404b.—Male, 32.5 cm. long. Left lung almost fully inflated; right airless. Stomach contains blood. Viscera normal in gross appearance. Both cultures containing heart's blood developed a mixed growth of several species. The liver culture remained sterile. In both tubes containing stomach contents growth appeared above the condensation water in 24 hours. On the 2nd day a heavy crop of minute colonies appeared on the slant, identified as pure cultures of *B. abortus*.

It appears from the foregoing protocols that bacilli resembling *Bacillus abortus* were isolated from the fetuses of 3 out of 4 sows. The fourth sow had a normal litter and was evidently not a carrier. Contrasting with bovine abortion, the distribution of bacilli in the fetuses was more general since cultures were readily obtainable from heart's blood and internal organs. In the bovine fetus the bacilli are in most cases limited to the digestive and pulmonary tracts and are sometimes wholly absent. The porcine fetuses examined showed no visceral lesions. The placentas obtained showed exudative processes resulting in thin excrescences. The nature of the peculiar fat-like expansions found on the placenta of No. 401 has not been elucidated.

Concerning the strains of *Bacillus abortus* obtained from the three sows or their litters, it may be stated briefly that they were identical so far as our comparative studies went. They all grew readily in

unsealed tubes and growth was visible within 2 to 3 days. The sealing had a distinctly retarding effect before or after the colonies had appeared. The rate of growth is best illustrated in the following experiment.

From a colony (401) a trace of growth is transferred on a platinum wire to 5 cc. bouillon and the latter thoroughly shaken. A loop of this suspension is transferred (*a*) to an agar slant which is sealed with sealing wax, and (*b*) to one having the lower end of the cotton plug just dipped in paraffin. To (*c*) unsealed the platinum wire transfers directly a trace from the colony. After 28 hours the bouillon suspension is distinctly clouded. (*c*) has a fairly good film. After 48 hours (*a*) and (*b*) are now covered with a dense layer of minute colonies. Those in (*b*) are 2 or 3 times the size of those in (*a*).

Pathogenic Action on Guinea Pigs.—Strain 401 isolated directly from a fetus was also recovered from guinea pigs as follows: The contents of a coil of the small intestine of fetus were suspended in bouillon and injected into two animals.

Guinea Pig 4180.—Weight 510 gm. Received subcutaneously 1 cc. suspension of fetal intestinal contents. Killed after 30 days. All subcutaneous lymph nodes swollen to about twice original dimensions. A necrotic focus in right kneefold node. A moderate number of minute grayish points showing under liver capsule. Spleen markedly congested. Dimensions twice normal. On section follicles irregularly enlarged. In two agar slants containing a bit of spleen, one sealed, growth appeared within 2 days in the unsealed tube. To hasten growth the sealed tube was pierced with a hot iron rod. 2 days later growth had begun in this tube above the bit of tissue.

Guinea Pig 4181.—Weight 470 gm. Chloroformed after 30 days. Lesions as in preceding animal. Cultures containing spleen bits show after 2 days about 100 characteristic colonies.

Two guinea pigs were later on inoculated with suspensions of pure cultures.

Guinea Pig 4221.—Received subcutaneously $\frac{1}{2}$ cc. of a 1/375,000 dilution of an agar slant. The dilution is prepared by washing the entire growth into the condensation water, which is equal to $\frac{1}{2}$ cc. This is further diluted to the titer indicated. Guinea pig killed after 26 days. Left kneefold and axillary nodes swollen and contain firm, yellow, 1 mm. foci. Other nodes inconspicuous. Spleen markedly congested. Dimensions twice normal. Minute grayish foci on section. Under liver capsule minute grayish foci. Cultures from spleen positive. Tubes contain several hundred typical colonies.

Guinea Pig 4209.—Receives the same culture but diluted only to 1/1250, into peritoneal cavity. Chloroformed in 25 days. Subcutaneous lymph nodes swollen. Spleen extremely full of blood. Dimensions $2\frac{1}{2}$ to 3 times normal. Cultures as in preceding case.

Bacillus abortus was isolated from the uterine swab of Sow 404. The lesions produced are like those of Strain 401.

Guinea Pig 4232.—Receives subcutaneously $\frac{1}{2}$ cc. of a turbid bouillon suspension of swab from Sow 404. Chloroformed in 21 days. At point of inoculation an abscess in the subcutis. Nearest kneefold lymph node, bean size, with still firm, necrotic foci. Spleen congested. Dimensions twice normal. Surface sprinkled over with minute grayish points. Spleen cultures contain several hundred colonies.

Guinea Pig 4231.—Receives 1 cc. of same suspension. Killed in 21 days. Lesions as in preceding animal with the addition of four small abscesses in omentum. Spleen and omentum cultures develop countless colonies. In cultures from both animals the growth in the sealed cultures was much retarded or else absent until seal penetrated and tubes reincubated.

Cultures of *Bacillus abortus* having identical characters in culture and in guinea pigs were thus isolated directly and through guinea pigs from 401 and 404. Material from 402 was not passed through guinea pigs.

Agglutination Reactions.—To determine the serological relationship between the swine strain and bovine types a culture recently isolated from a case of bovine abortion and serum from an old bovine carrier of *Bacillus abortus*, probably in the udder (No. 930), were used. The cow had carried agglutinins in her blood for a number of years. The swine sera were obtained by bleeding the sows from the tail. The absorption tests were performed as follows:

A Blake bottle containing nutrient agar was inoculated and incubated 48 hours. A suspension in normal salt solution was made which was 11 times the density of 2.4 on the Gates scale. 1 cc. of serum was mixed with 4 cc. of the suspension and centrifuged. The deposit was stirred up, incubated 2 hours, and centrifuged. The clear serum in a 1/5 dilution was tested in higher dilutions as indicated in Table I.

Reciprocal agglutination and absorption tests with a strain from swine are given in Table I.

The irregular inhibition of agglutination shown in this table is also evident in tests to be reported below. The serum of the cow is equally, but not completely, absorbed by cow and pig strain.

In Table II are recorded additional direct and cross-agglutination tests. Certain irregularities are also evident in these data. The serum from a normal pig was free from agglutinins towards swine strains. A test not tabulated showed complete absence of agglutinins

TABLE I.
Serum of Cow 930.

Strains of <i>B. abortus</i>	Dilutions									Controls (salt solution)
	1/10	1/20	1/40	1/80	1/160	1/320	1/640	1/1280	1/2560	
Cow 1373.....	C	++++	++++	C	C	+++	+	-	-	-
Swine 402.....	+++	+++	++++	C	++++	++	-	-	-	-

Absorption Test.

(Absorbed with Strain Swine 402)	Serum of Cow 930									
Cow 1373.....	C	+	-	-	-	-	-	-	-	-
Swine 402.....	C	+	-	-	-	-	-	-	-	-

TABLE II.
Cross-Agglutination Tests (Cow and Swine Sera).

Serum of	Serum dilutions									Control	Strain used for aggluti- nation
	1/10	1/20	1/40	1/80	1/160	1/320	1/640	1/1280	1/2560		
Normal pig.	-	-	-	-	-	-	-	-	-	-	Sow 401
Cow 930....	C	++++	+++	++++	++++	++++	+++	-	-	-	" 401
Swine 462...	++++	++	+++	C	C	C	+++	+	-	-	" 404
Cow 930....	++++	++++	++++	++++	++++	+++	-	-	-	-	" 404
Normal pig.	-	-	-	-	-	-	-	-	-	-	" 404
Swine 461...	++++	C	C	C	C	C	++++	+	-	-	<i>B. abortus</i> 1373
" 461...	C	C	C	C	++++	++++	+++	-	-	-	Sow 402

towards the cow strain in dilutions as low as 1/10. The additional sows included in this and the following test are from the infected herd. No. 460 is a young pregnant sow, evidently not infected. No. 461 is also a young pregnant sow, showing in her serum a high agglutinin content. No. 462 is an old sow, also with a high agglutinin titer.

In Table III are given comparative tests of the swine sera with strains from swine and Cow 1373.

Here again the serum No. 460 appears free from specific agglutinins. The blood of infected Sow 402 is low in agglutinins. The rest are high. Both cow and swine strain show the irregular inhibitions with the maximum agglutination and subsidence of the bacteria around 1/160.

TABLE III.

Agglutination of Strains from Cow 1373 and Sow 401 by Various Sera from Infected Herd.

Source of swine sera	Serum dilutions									Control	Strain used
	1/10	1/20	1/40	1/80	1/160	1/320	1/640	1/1280	1/2560		
B	++	++	++++	C	C	C	C	+	-	-	Sow 401
C	++	++	C	C	C	C	++	-	-	-	" 401
402	++	++	++	++	-	-	-	-	-	-	" 401
	++	+	=	++	+	-	-	-	-	-	Cow 1373
404	++	++	+	++++	C	++++	+	-	-	-	Sow 401
	++	++	++	++++	C	++++	-	-	-	-	Cow 1373
460	+	+	=	+	+	-	-	-	-	-	Sow 401
	=	=	-	++	++	-	-	-	-	-	Cow 1373
461	++	++	++++	++++	C	C	C	++++	++	-	Sow 401
	++	++	++++	++++	C	C	C	++	-	-	Cow 1373
462	++	+	+++	C	C	C	++++	+++	-	-	Sow 401
	+	+	+++	C	C	C	++++	+	-	-	Cow 1373

The Effect of Feeding Cultures from This Outbreak on Swine.—Only one experiment has been completed to date.

Sow 344, born June, 1925. Last litter May 4, 1927. Late in September the sow was put into an outdoor enclosure provided with a small house for shelter on soil not overrun by swine for at least 13 years. A Blake bottle containing an agar layer was seeded with strain from Swine 401, now 5 months under cultivation, and incubated for 2 days. On Oct. 8, 1927, the sow was fed with the salt solution suspension of the entire culture mixed with dissolved powdered milk.

The sow gave birth to two normal young on October 19. Placental material was not recovered nor any material indicating the birth of dead pigs. The agglutinin reactions of the blood of the sow on the day of feeding and the day following farrowing are given in Table IV.

Following the farrowing, a swab was inserted into the uterus and the attached material suspended in normal saline and injected into the peritoneal cavity of two guinea pigs. Both were chloroformed after 46 days. Autopsy and cultures were negative. At the same time two guinea pigs received an intraperitoneal injection of the sow's milk. Both, killed after 46 days, had large spleens and other lesions diagnostic for *Bacillus abortus* infection. Pure cultures of *Bacillus abortus* were obtained from spleen tissue. The guinea pig lesions

TABLE IV.
Agglutination Reactions of Sow before and after Infection.

Blood drawn	Serum dilutions								
	1/10	1/20	1/40	1/80	1/160	1/320	1/640	1/1280	1/2560
October 8.....	++++	++++	C	++++	+	±	-	-	-
" 20.....	++++	+++	++++	C	C	+++	+	±	-

resembled closely those produced originally by the culture fed. The significant result of this experiment is contained in the fact that the organism fed became located in the mammary gland and not in the uterus of the pregnant sow. The agglutinin test before infection suggests possible infection of the sow with *Bacillus abortus*, but tests on other sows of the herd yielded nearly the same figures. The herd has been free from abortion and recent tests of the milk from four sows on guinea pigs were entirely negative.

In the spring of 1928, the attention of this Department was called to an outbreak of swine abortion in another State institution.⁴ The sows gave birth to large litters of which most died on the same day. Material from uterine swabs and sow's milk were injected into a large number of guinea pigs. All of these when killed 4 to 6 weeks later

⁴The writer is indebted to Dr. Henry W. Dustan for material from this outbreak.

were normal and cultures of the entire, small spleen negative. Similarly cultures made from the organs of four new-born pigs were negative.

In this outbreak *Bacillus abortus* evidently played no part. The work is mentioned here so that those entrusted with the investigation of such outbreaks on the spot do not fail to consider causes other than infection with *Bacillus abortus*.

SUMMARY.

The outbreak of infectious abortion in swine, probably the first reported from the eastern United States, was associated with a strain of *Bacillus abortus* growing rapidly on ordinary nutrient agar slopes without seal and presenting certain slight pathological deviations from the bovine form of disease in guinea pigs such as the occurrence of necrotic, suppurating foci in spleen and lymph nodes. Agglutination tests, comprising both cross-agglutination and absorption procedures failed to distinguish the strain from the bovine type. The gross appearance of the fetuses from this outbreak was normal. The shreds of placentas obtainable indicated slight erosion of the chorionic epithelium and some exudation. The specific bacilli were quite widely disseminated in the tissues of the fetuses. The pathogenic action of this swine strain on guinea pigs was evidently much feebler than that of most earlier swine strains as reported and it approached more closely that of bovine strains. The culture fed to a pregnant sow failed to produce abortion, possibly because of the advanced stage of pregnancy. The organism was not recovered from the uterus but was found in the sow's milk.

THE REACTION OF HUMAN BILE AND ITS RELATION TO GALL STONE FORMATION.

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Little is known concerning the chemical changes occurring in bile which lead to the formation of calculi, even though the problem of gall stone formation has attracted a great deal of attention over a long period. This absence of knowledge is especially evident with respect to the acid-base equilibrium of the bile. The suggestion has been made repeatedly that the concentration of hydrogen ions may be one of the factors which governs the solubility of calcium and the bile pigments. In addition, some evidence exists that the solubility of the cholesterol is influenced by the reaction of the bile.

Of the few researches that have dealt with the hydrogen ion concentration of bile, only those of Drury, McMaster and Rous (1) and of Stern (2) have considered its possible effect upon gall stone formation. The work of the former confirmed the observations of Okada (3) and of Neilson and Meyer (4) that bile from the gall bladders of animals was more acid than bile which had been freshly secreted by the liver. They found similar differences in human specimens, and observed further that the acidity of dog bile increased progressively with the time of its stay in the gall bladder. A study of the calcium concentration showed that it rose and fell with the hydrogen ion concentration and that calcium carbonate and cholesterol were precipitated from alkaline bile, whereas acidification prevented the precipitation. Neilson and Meyer had previously found that the diseased gall bladder of the rabbit was unable to change the reaction or the concentration of the bile. On the basis of these observations, Drury, McMaster and Rous suggested that one of the functions of the gall bladder was to acidify the bile and in this way increase its solvent power for calcium and perhaps cholesterol. Failure of the gall bladder to function in making the bile more acid,

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particularly while it is being concentrated, presumably would be followed by precipitation and possibly by calculus formation. Recently Drury and Rous (5) attempted to test this hypothesis experimentally by altering the gall bladders of dogs and rabbits so that the bile would not become more acid during its storage in the gall bladder. In one case gall stones formed, although the experiment was complicated by bacterial infection. The work of Stern (2) is based on his study of cholesterol hydrosols. It is his belief that gall stones are formed because of an abnormally increased hydrogen ion concentration in the bile; however, his attempts to form concretions by massive administrations of acid to animals were unsuccessful.

Before either of the hypotheses just described is accepted more direct evidence is needed. In our work, therefore, the hydrogen ion concentration of human bile has been studied in order to ascertain whether gall stones are associated with a change in its reaction. In connection with this study additional evidence was obtained relative to the action of the gall bladder in modifying the pH in the bile.

Method.

The specimens were aspirated from the gall bladder *in situ* at operation or immediately after its removal with the clamps left in position on the cystic duct.¹ A syringe was used for withdrawing the specimen. Exposure to air with subsequent loss of CO₂ was avoided.

Fistula bile was collected under mineral oil, care being taken to keep the drainage tubes filled with bile and free from air bubbles. It was difficult to do this and several experiments were invalidated because of changes in the bile brought about in this way. Our experience in collecting bile from fistulæ suggests that the more alkaline values commonly reported for fistula bile may be the result of unsuspected losses of CO₂ within the drainage system. The use of mineral oil to prevent gas exchange was not satisfactory because the loss of CO₂ was not completely prevented, but no better method was available for making

¹ The preoperative treatment of the patients was the same in all cases except one (noted below). A light meal was given at 5 p.m. on the preceding day, following which the patient received no other food until after the operation. An enema was given in the morning and was followed, 30 minutes before operation, by the usual injection of morphine and atropine sulfates. All of the operations were done in the morning, hence the collection of the bile had been preceded by 15 to 19 hours of fasting.

The only deviation from this procedure was made in a case of diabetes (Table II, Feb. 27, 1928) when glucose and orange juice were given with 10 units of insulin 1½ hours before operation.

continuous collections over many hours. In a few instances the bile was collected in sampling tubes over mercury. By comparing these specimens with some taken under oil, the error caused by the use of the latter was determined and the corresponding correction applied. The maximum change was 0.005 pH per hour of exposure to the mineral oil. The specimens from the gall bladder were tested at once and did not need correction.

The pH was determined by the use of the quinhydrone electrode. When enough material was available the results were checked by means of the hydrogen electrode. The refill technique described by Cullen (6) was used with the latter. Quinhydrone electrode determinations were made by means of the apparatus and procedure described by Meeker and Reinhold (7). Results by the two methods were practically identical. The average of all determinations by the quinhydrone electrode was 0.015 pH less than the average of the hydrogen electrode determinations on the same specimens. The maximum differences were +0.01 pH and -0.05 pH. Some drift of the potentials occurred when the quinhydrone electrode was used in bile, but at a much slower rate than in the case of similar determinations in blood plasma. Error from this source was overcome by reading the potential as quickly as possible after mixing the quinhydrone into the sample. It had been demonstrated previously that this precaution eliminated the error due to drifting when the method was used for plasma pH determinations.

EXPERIMENTAL DATA.

The hydrogen ion concentration of human bile from the gall bladders of unobstructed, calculus-free biliary systems varied between pH 7.10 and 7.30. The figures are given in Table I. There were no apparent pathological changes except that three of the gall bladders showed adhesions. Possibly it is significant that the bile from gall bladders with adhesions was slightly more alkaline than the other specimens in this group. Two of the gall bladders showing these changes were tested by the Graham method. Both functioned normally, so far as ability to concentrate or evacuate the bile was concerned.

No data for the hydrogen ion concentration of normal human gall bladder bile were discovered in the literature. Labbé, De Moor and Nepveux (8) determined the pH colorimetrically of diluted bile obtained by non-surgical drainage of the gall bladder by means of the Meltzer-Lyon technique. Their specimens varied between pH 6.6 and 7.4. Contamination by the contents of the duodenum was inevitable in samples collected in this way.

The specimens from gall bladders in which calculi were present were classified according to the presence or absence of obstruction of the bile

ducts. The data on unobstructed biliary systems are presented in Table II. With two exceptions the pH values were either above or below the normal limits established in Table I. Four of the fifteen specimens were slightly more alkaline than the highest of the normal group, but a fifth was 0.40 pH more alkaline. Eight were more acid. The differences measured from the lowest normal figure, were 0.01 pH to

TABLE I.

The Reaction of Human Gall Bladder Bile in the Absence of Concretions or Obstruction.

Date	Number	Bile pH 37.5°C.	Culture	Remarks
1927				
Apr. 20	*10693	†7.19		Carcinoma of the stomach
May 18		7.24		Adhesions around gall bladder
Dec. 2	12252	7.10	Negative	
1928				
Jan. 15	12733	7.13	Negative	
Apr. 16	13266	7.22	Negative	†Gall bladder gave normal response to test of function. Pericycstic adhesions
23	13317	7.26 7.31	Negative	Normal gall bladder function. Adhesions

* Index number of the case history in the records of the University of Pennsylvania Hospital.

† Emptying and concentrating power of the gall bladder as determined by the Graham method.

‡ The patients had fasted 15 to 19 hours. The preoperative treatment was the same as in the cases with gall stones or obstruction.

0.22 pH. The direction or the size of the change in reaction was not related to the type of the stone found, nor were there any systematic or consistent differences which could be related to the presence of calculi. In view of the small number of specimens which were obtained from calculus-free gall bladders, some of the preceding probably represent unchanged values. Slight obstruction of the cystic duct, unde-

TABLE II.

The Reaction of the Human Gall Bladder Bile When Concretions Were Present and Obstruction Was Absent or Slight.

Date	Number	Bile pH 37.5°C.	Concretions		Culture	Remarks
			Number	Type*		
1927						
May 27	11106	7.42 7.42	+	Cholesterol pigment	Negative	Subacute cholecystitis. Acute pancreatitis. Gall bladder function poor
June 20	11155	7.32 7.32	++	Cholesterol pigment		
Oct. 19	12236	6.88 6.92	24	Cholesterol pigment		Chronic interstitial cholecystitis. Gall bladder function normal
28	11999	6.94	8	Cholesterol pigment	<i>Staph. hæmolyticus.</i> Diphtheroids	Chronic interstitial cholecystitis. Cholesterol deposits on gall bladder wall
Nov. 2	12051	7.26 7.33 7.34	20	Cholesterol pigment	Negative	Chronic interstitial cholecystitis. Cholesterol deposits. Adhesions to duodenum
14	12263	7.04† 7.06†	1	Cholesterol pigment		Acute hemorrhagic cholecystitis. Edema
Dec. 2	12266	6.97 6.97	+++	Cholesterol		Acute pancreatitis. Gall bladder normal
1928						
Jan. 15	12563	7.18	16	Cholesterol pigment	<i>Staph. albus</i> non-hemolytic	Chronic interstitial cholecystitis
Feb. 27	13134	7.09	1	Cholesterol (crystals). No pigment		Cholesterol deposits on gall bladder walls. Function poor

* The classification of calculi in this and other tables is made on the basis of physical appearance. Chemical analyses were not made.

† Contaminated with blood.

TABLE II—*Concluded.*

Date	Number	Bile pH 37.5°C.	Concretions		Culture	Remarks
			Number	Type		
1928						
Feb. 27	13034	7.00	40-50	Cholesterol pigment	Negative	Chronic interstitial cholecystitis. Gall bladder nearly full of stones
Mar. 29	13044	6.99	+	Calcium pigment	Negative	Gall bladder had normal appearance
30	13261	7.07	11	Cholesterol	Hemolytic <i>Staph. aureus</i>	Subacute interstitial cholecystitis. Cholesterol deposits in gall bladder
Apr. 22	13708	7.68 7.70	++	Cholesterol pigment		Subacute cholecystitis
May 14		6.95 6.96	++	Cholesterol	Negative	Gall bladder apparently normal
June 6		7.35	+		Negative	Chronic cholecystitis

tected at operation, may have existed in others and must also be considered in explaining these observations.

In the presence of obstruction the acidity of the gall bladder contents was increased (Table III). Complete obstruction of the common or cystic duct was characterized by pH values near 6.40. When the obstruction was only partial the pH was higher. An exception occurred in a case of obstruction caused by a carcinoma of the head of the pancreas (Table III, July 8), in which the gall bladder was almost completely atrophied. The specimen, taken from the greatly enlarged common duct, was pigment-free. Its pH was 7.30. Apparently the obstructed bile was not acidified by the liver; otherwise in this instance the usual fall in pH would have occurred. Instead it was dependent on the presence of the gall bladder and must have happened within the latter organ. Neither was the increased acidity a result of mechanical irritation by concretions in the gall bladder. Table III

TABLE III.

The Reaction of Bile from the Human Gall Bladder Following Obstruction of the Common or of the Cystic Ducts.*

Date	Number	Bile pH 37.5°C.	Concretions		Culture	Remarks
			Number	Type		
1927						
June 27	11338	6.37 6.40	3	Cholesterol pigment	Negative	Pigment-free "white bile." Cystic duct obstruction. Acute cholecystitis
July 8	11291	7.30	0			Carcinoma of head of pancreas. Gall bladder atrophied. Specimen taken from cystic duct. Pigment-free
Nov. 8	12068	6.82 6.83	+	Cholesterol pigment	<i>B. coli communis</i>	Acute cystic duct obstruction. Marked edema. Specimen practically colorless
17	12126	6.44	+	Cholesterol pigment	Diphtheroids, <i>Staph. albus</i>	Chronic cholecystitis. Complete obstruction. Specimen was colorless
16	12340	6.77 6.79	40	Cholesterol pigment	Negative	Subacute interstitial cholecystitis. Partial obstruction
16	12135	6.70	+	Cholesterol pigment	<i>B. coli communis</i>	Nearly complete obstruction. Edema. Bile contaminated slightly with pus
30	12257	6.79	2 Many crystals	Cholesterol	Negative	Acute cholecystitis Partial edematous obstruction
1928						
Feb. 22	12830	6.61	1	Soft sediment. Cholesterol crystals, pigment	<i>B. coli communis</i>	Chronic and acute cholecystitis. Thickened diseased gall bladder with adhesions. Partial obstruction of cystic duct

* Unless stated to the contrary the site of obstruction was in the common duct.

TABLE III—*Concluded.*

Date	Number	Bile pH 37.5°C.	Concretions		Culture	Remarks
			Number	Type		
1928						
Mar. 7	13092	6.43	4	Cholesterol	Negative	Chronic interstitial cholecystitis. Partial cystic duct obstruction. Gall bladder walls thickened. Adhesions. Pale yellow bile
23	13098	6.70	2	Cholesterol		Acute cholecystitis. Incomplete obstruction. Bile had light green color
29		6.60	+	Tar-like sediment	Negative	Carcinoma of papilla of Vater. Nearly complete obstruction. Yellow incrustation on gall bladder wall. Yellow bile, low pigment concentration
Apr. 23	13578	6.40	12	Cholesterol pigment	<i>B. coli communis</i>	Complete obstruction of cystic duct
June 4		6.87	36	Calcium pigment	Negative	Partial obstruction of cystic duct. Thickened gall bladder wall with lesions

includes a case (Mar. 29), in which the common duct was blocked by a carcinoma of the papilla of Vater. The gall bladder appeared to be normal and no concretion was present, although a small amount of sediment had collected in the fundus. The pH of this bile was 6.60, a figure which corresponded well with those obtained when the same degree of obstruction had been brought about by gall stones.

The result of the obstruction so far as change in the acidity of the bile was concerned, did not differ when the site of the obstruction was in the cystic duct. In either event the effect would be to oppose the expul-

sion of the contents of the gall bladder while interfering to a lesser extent with the flow of bile into the gall bladder. As a consequence the time during which the bile was acted upon would be lengthened and changes in composition accentuated. The higher acidity of the contents of the completely obstructed gall bladder, therefore, can be explained best as being a result of the prolonged exposure to the action of this organ. These specimens were characterized by low concentrations of pigment and solids. The viscosity was greatly increased.

The acidification of the bile was not related to the presence of microorganisms. More than 50 per cent of the pathological specimens were sterile, yet gave results identical with those obtained when infected specimens were studied.

Fistula Bile.—Additional evidence concerning the changes in the reaction of human bile was secured by collecting simultaneously samples of bile from the gall bladder and from the common duct. Bile from the gall bladder was obtained from a drainage tube inserted into the fundus. Common duct bile was collected from a common duct fistula.²

A sufficient number of specimens was obtained to show that after an initial period of recovery from the operation, the bile from the gall bladder was definitely more acid than that from the common duct (Tables IV and V). Shortly after the operation the difference was small or non-existent. As the condition of the patient returned toward normal, the bile from the gall bladder remained at the same pH or increased slightly, while the pH of the common duct bile increased appreciably. Finally when a light general diet had been resumed and recovery was well under way, the bile from the former was more acid than the latter by from 0.04 to 0.24 pH. Because of the smaller output of the gall bladder fistulae and the longer drainage tubes required, the probability of error due to loss of CO₂ was greater for these specimens than for those from the common duct fistulae. Consequently such errors would diminish the difference between the hydrogen ion concentrations of the bile from the two sources and would tend to obscure the changes which had taken place.

² The collection of bile was made under approximately sterile conditions. Together with the short intervals at which the specimens were collected this should have eliminated any significant error which might have been caused by bacterial action.

TABLE IV.

The Reaction of Bile Collected Simultaneously from Common Duct and Gall Bladder Fistulae.

Date	Time	Gall bladder bile		Common duct bile			Diff.	
		Volume	pH	Volume	pH	Avg. pH for 24 hrs.	pH	
1927	hrs.	cc.	38°C.	cc.	38°C.			
Nov. 16			6.78					*No 12340 Liquid diet
17-18	12†			145	6.86			Night
18	12			140	6.89			Day
18-19	12			130	6.90			
19	12	68	6.95	117	6.95	6.92	+0.03	
19-20	12			85	6.95			
20	12	65	6.96	146	7.11	7.05	-0.09	
20-21	12			132	7.06			
21	12	68	6.96	188	7.19	7.14	-0.18	Soft diet
21-22	12			159	7.19			
22	12	85	7.00	219	7.27	7.24	-0.24	
22-23	12			85	7.26			
23	12		7.08	155	7.22	7.23	-0.15	Drainage tubes clamped 12 hrs. Plasma pH 7.39
23-24	12			107	7.23			
24	12		7.08	65	7.26	7.24	-0.16	Drainage tubes clamped 12 hrs.
25	12			58	7.35			
25-26	12	53	7.14	45	7.35	7.35	-0.21	Drainage tubes clamped 12 hrs. Plasma pH 7.38
28	$\frac{1}{2}$				7.27			Before breakfast
	$2\frac{1}{2}$				7.43			After breakfast
	$\frac{1}{2}$				7.31			After preceding
29	$\frac{1}{2}$				7.37			Before breakfast
	5				7.35			After breakfast
	$\frac{1}{2}$				7.39			After preceding

* See Table III, Nov. 16.

† The specimens were collected over intervals varying between 2 and 6 hours. In order to save space and to make the data more accessible the results have been expressed in terms of 12 hour periods beginning at 8 a.m. and 8 p.m. The same applies to Table V.

The ingestion of a test meal in one instance was followed by an alkaline tide in the bile during a $2\frac{1}{2}$ hour period. A similar experiment on the following day, in which the collection of the postprandial specimen was extended over 5 hours, failed to show a significant change.

TABLE V.
The Reaction of Bile from Common Duct and Gall Bladder Fistulae.

Date	Time	Gall bladder bile		Common duct bile			Diff.	
		Volume	pH	Volume	pH	Av. pH for 24 hrs.		
1927	hrs.	cc.	33°C.	cc.	38°C.			
Nov. 17			6.44					*No. 12126
18								
19	12			60	6.83			Day
19-20	12				6.81			Night
20	12			215	7.01			Liquid diet
20-21	12			140	6.96			
21	12			155	6.97	7.00		
21-22	12	11	6.94	139	7.03		-0.06	
22	12			95	7.03	7.07		Soft diet
22-23	12	61	6.97	150	7.10		-0.10	
23	12			123	6.94	7.01		Drainage tubes clamped 6 hrs.
23-24	12	21	6.97	171	7.06		-0.04	
24	12			153	7.03			Plasma pH 7.34
24-25	12				7.06			
25	12		6.98	75	7.06		-0.08	

* See Table III, November 17.

The reaction of the common duct bile in the few comparisons made was slightly more acid than that of the blood.

DISCUSSION.

Under certain conditions marked changes occurred in the hydrogen ion concentration of the gall bladder bile. From the data presented

it is apparent that these were not associated with the presence of concretions in the gall bladder. While deviations from the "normal" reaction were observed in almost every instance when gall stones were present, it seemed improbable that these changes were of sufficient size to influence solubility relationships. However, it was possible that the formation of calculi was caused by transitory changes in the acid-base balance of the bile or by a succession of such changes. It would then be unlikely that more than a few of the specimens in Table II would vary much from the normal values. Actually only one specimen showed a large difference, although six others differed from the lowest or highest normals by more than 0.10 pH. The acid character of several specimens of this group was probably the result of the same causes which increased the acidity of the bile during obstruction.

The pronounced increase in the acidity of bile following obstruction of the ducts coincided with the observations made by Drury, McMaster and Rous (1) on the pH of the gall bladder bile of dogs in which stasis had been induced experimentally. Obstruction increased the time during which the bile was exposed to the action of the gall bladder. If the latter was able to acidify its contents, those specimens which had been exposed to its action for a long time would necessarily have become more acid than those which had been expelled with less delay. Consequently complete obstruction was accompanied by the greatest acidification. Several additional facts supported the conclusion that it was the gall bladder which brought about the change in the reaction of the bile. Most conspicuous was the failure of the biliary system to acidify the bile when the gall bladder was absent (atrophied). Of even greater significance was the consistently higher hydrogen ion concentration in bile from a gall bladder fistula when compared with that from a common duct fistula, collected over the same time interval. That the changed acidity was not the result of the irritating action of the calculi was shown by the occurrence of identical changes in obstructed calculus-free gall bladders. Infection was ruled out by similar reasoning.

While it seemed quite certain from the foregoing that the gall bladder altered the hydrogen ion concentration of the bile, this should not be allowed to obscure the possibility that the acid bile found in the gall bladder following obstruction was secreted as such by the liver either

as a result of damage to that organ or in an attempt to increase the solvent power of the bile. The acidity of bile coming through a common duct fistula from the liver was greatest after severe obstruction of the common duct, and some of this acid secretion must have entered the gall bladder. In cystic duct obstruction, however, there was no injury to the liver and the secretion and composition of bile from the liver presumably was not affected. Since the change in reaction of the gall bladder contents was the same in direction and degree after obstruction of the cystic duct as it was after common duct obstruction, the changes which occurred must have been accomplished by the gall bladder.

No evidence is available concerning the means by which the contents of the gall bladder are made acid. Absorption of material from the bile by this organ as shown by a decrease in solids (unpublished data) is one probability, while secretion by the gall bladder as reflected by the increased viscosity of the contents likewise may have had a part in the changes which occurred. Whatever the mechanism, it is apparent that even marked pathological changes did not prevent it from functioning. The significance of the variations in the hydrogen ion concentration of the bile remains obscure. It is hoped that solubility studies now in their preliminary stages in this laboratory will assist in establishing their relation to calculus formation.

SUMMARY.

1. The human gall bladder acidifies the bile. In this respect its action is similar to that of the gall bladders of lower animals, previously described by other workers.
2. The hydrogen ion concentration of gall bladder bile is increased considerably in cases of obstruction of the common or cystic ducts. The highest values were found following complete obstruction.
3. The occurrence of gall stones was not associated with a consistent change in the hydrogen ion concentration of the gall bladder bile.

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DIFFERENTIATION BETWEEN SOME TOXIC SUBSTANCES IN ANAEROBICALLY PRODUCED AUTOLYSATES OF PNEUMOCOCCI (TYPES I AND II).

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In preceding papers (1, 2), it was shown that anaerobically produced autolysates of pneumococci cause necrosis when injected intradermally, and marked lung lesions and death when injected intratracheally, into small guinea pigs. In the present study, I wish to describe experiments made to determine whether or not the necrotizing and the lung-toxic effects are due to the same or to different substances present in these autolysates.

EXPERIMENTAL.

Preparation of the Poisonous Autolysates.

The method of preparation of the toxic autolysates has been described in detail in previous papers (1, 2). Briefly, the method is to autolyze in fresh broth at room temperature under vaseline seal, the centrifuged pneumococci obtained after 18 hours growth on double strength veal infusion broth which contains 4 per cent Witte peptone. The Berkefeld filtrates of these autolysates are toxic for both the skin and the lungs of young guinea pigs and both these toxic effects can be preserved without deterioration when such filtrates are kept in the ice box under vaseline seals.

Heat Lability of the Toxic Autolysates for the Lungs of Guinea Pigs.

In a previous article it was shown that the necrotizing principle was completely destroyed when heated to 60° for 5 minutes under vaseline seal. It was found that under these same conditions the lung-toxic principle was also destroyed. Guinea pigs injected intratracheally with 0.2 cc. of the heated autolysate did not appear sick at any time and did not die spontaneously. Several of these pigs were

killed with ether 1 to 3 days after the injection of heated poison, and all showed at autopsy a small red patch of consolidation in one or more lobes of the lungs near the hilum, but never the diffuse lesion caused by the unheated autolysates.

Sensitiveness to Oxidation of the Toxic Autolysates for the Lungs of Guinea Pigs.

It was noted in a previous paper that the skin-necrotizing poison in the autolysates was extremely sensitive to oxidation and deteriorated rapidly when exposed to the air. We found that the lung-toxic substance also weakened quickly after removal of the vaseline seal, even when the tubes containing the preparations were kept in ice water. A few experiments were made with aerobically prepared autolysates,—autolysates prepared exactly like the anaerobic autolysates, except that the pneumococci were autolyzed without the vaseline seal and the filtrates preserved in the ice box were also without a vaseline seal. These aerobically prepared autolysates produced no skin reaction when injected intradermally and no symptoms or death when injected intratracheally into young pigs. In some pigs killed after the injection of aerobic autolysates, small areas of consolidation near the hilum were noted, similar to those found after the injection of heated autolysates.

Is the Lung-Toxic Product Neutralizable by the Anti-Autolysate Serums Which Neutralize the Necrotizing Principle?

It was shown previously that there was an apparent neutralization of the necrotizing principle when necrotizing autolysates from either *Pneumococcus* I or II were mixed *in vitro* with the serum of rabbits immunized against *Pneumococcus* I autolysates. It was important to determine whether the substance or substances which were responsible for the death of guinea pigs injected intratracheally with the toxic filtrates could also be neutralized by the antisera which neutralized the necrotizing principle.

The tests for neutralization of the lung-toxic product were set up in a way similar to our former tests for neutralization of necrotizing substances. These tests were carried out as follows: 0.9 cc. of a well chilled necrotizing filtrate was placed in

each of three narrow test-tubes. To the first tube was added 0.1 cc. of the anti-serum; to the second tube 0.1 cc. of normal rabbit serum; and to the third the same amount of broth. The contents of the tubes were well mixed, and a heavy vaseline seal was then added to each tube. After standing at room temperature for 1 hour, the tubes were again chilled, the vaseline seals removed, and the preparations in amounts of 0.25 cc. were injected intratracheally into small guinea pigs of approximately equal weights. The results of these experiments were clear-cut. The pigs which received the toxic autolysate and immune serum showed no symptoms and survived. The other two pigs died with the usual symptoms and the autopsy findings were typical. This experiment has been repeated several times and always with the same results.

TABLE I.

	Toxicity for guinea pigs' lungs. 0.2 cc. injected intra- tracheally	Toxicity for guinea pigs' skin. 0.1 cc. injected intra- cutaneously
Necrotizing filtrate.....	+++	+++
Adsorbed with red cells.....	0	+++

These experiments have been repeated with two potent anti-autolysate horse serums. These serums in amounts of 0.0002 cc. neutralized at least one lethal dose of the lung-toxic autolysates, *viz.*—0.1 cc. of these serums diluted 1–100 neutralized 0.9 cc. or more than five lethal doses of the toxin. (These last experiments will be given in detail in a later publication.)¹

From the fact that the anti-autolysate serum prepared with Type I neutralized the lung-toxic action of the autolysates of both *Pneumococcus* Type I and Type II, it is evident that the lung-toxic poison, like the pneumococcus necrotizing poison, is antigenically similar for both Types I and II and is therefore not type-specific.

These experiments seem to show that the necrotizing and lung-toxic poisons in the anaerobic autolysates are similar in their sensitiveness to heat and to oxidation and in their ability to be neutralized by the same anti-autolysate serums.

¹ These horses were immunized to the autolysate filtrates at Eli Lilly Co., in Indianapolis, under the supervision of Mr. W. A. Jamieson.

Toxicity of Red Cell-Adsorbed Autolysates for the Lungs of Guinea Pigs.

In a preceding paper, it was shown that necrotizing filtrates from which the hemotoxin had been removed by adsorption with red cells, were just as toxic for the skin of guinea pigs as untreated autolysates. It seemed of interest to determine whether or not such treatment had any effect on the toxicity of these autolysates for guinea pig lungs.

Accordingly guinea pigs of approximately equal weight were injected intratracheally with 0.2 cc. of necrotizing filtrates or the same filtrates which had just previously been adsorbed with red cells. Six different autolysates were tested in this manner. All the guinea pigs which received the untreated autolysates died with characteristic symptoms and autopsy findings, while none of the pigs which were inoculated with adsorbed autolysates showed any symptoms or died. Five of the latter pigs were killed with ether from 1 to 3 days after the injection, and at autopsy showed very much the same picture grossly as the pigs which had been injected intratracheally with the heated toxin described before. None showed the diffuse pneumonia regularly found in control animals. The consolidation about the hilum was, however, more extensive than in the pigs given heated toxin. All autolysates, both treated and untreated, were tested each time for necrotizing activity by inoculation into guinea pigs' skin and always proved to be equally toxic for the skin. These last experiments are summarized in Table I.

It is evident from these experiments that the substance or substances in the necrotizing autolysates, which when injected intratracheally bring about marked symptoms and death of guinea pigs, had been removed or inactivated by treatment with the red cells. An important question to be answered was whether or not the toxic substance for the lungs which had been removed was the pneumococcus hemotoxin, which is known to be adsorbed by red cells.

Toxicity of Pneumococcus Hemotoxin for the Lungs of Guinea Pigs.

To investigate this point, we produced pneumococcus hemotoxin by the freezing and thawing method of Avery and Neill (3) and inoculated the filtrates of these extracts, which were strongly hemolytic, intratracheally into guinea pigs. As a general rule, these hemotoxin preparations caused no symptoms and only slight lung involvement when injected intratracheally. However, we have obtained two preparations which were toxic for the lungs of guinea pigs, causing a diffuse lesion similar both macroscopically and microscopically, to that

caused by a weak lung-toxic autolysate. It is probable, therefore, that the pneumococcus extracts do occasionally contain the lung-toxic poison in considerable amounts. That the lung-toxic action of our autolysates is not due to the hemotoxic properties of the hemotoxin itself seems probable from the fact that no evidence of hemolysis was seen in the sections of the lungs of the pigs injected with the toxic autolysates and that the lung-toxic action of an autolysate may be neutralized by quantities of anti-autolysate serums which have no effect on the hemotoxin. (This last point will be brought out in greater detail in a later paper.) We have also found no definite parallelism in our autolysates between the toxic action for lungs of

TABLE II.

Toxic products in anaerobic autolysates	Present in aerobic autolysates	Present in anaerobic autolysates	Present in frozen and thawed pneumococcus extracts	Present in anaerobic autolysates adsorbed with red cells
Hemotoxin.....	0	+	+	0
Skin-toxic.....	0	+	0	+
Lung-toxic.....	0	+	±	0

guinea pigs and the amount of hemotoxin such autolysates contain. We think it probable, therefore, that the pneumococcus hemotoxin present in the autolysates is not responsible for either the dyspneic symptoms, or the death with marked lung lesions of guinea pigs injected intratracheally with the toxic autolysate, but that preparations of hemotoxin, produced by the freezing and thawing method, may contain definite amounts of the lung-toxic product.

It seems clear, therefore, that although the necrotizing and lung-toxic principles in the autolysates are similar in several respects, they differ in the important point that the necrotizing poison is not inactivated or adsorbed by red cells as is the lung-toxic principle. This fact appears to prove that the necrotizing and lung-toxic principles are separate entities. These facts are summarized in Table II.

CONCLUSIONS.

The necrotizing and lung-toxic principles present in certain anaerobically prepared autolysates of *Pneumococcus* Types I and II are similar in respect to extreme sensitiveness to heat and to oxidation, and to their ability to be neutralized by the same anti-autolysate serums.

These two poisons differ, however, in their ability to be adsorbed or inactivated by red cells; the lung-toxic principle being adsorbed or inactivated by such procedure while the necrotizing principle is not.

Since pneumococcus hemotoxin is present in the anaerobic autolysates and is also adsorbed by red cells, it seemed possible that it was this substance in the autolysates which caused the diffuse lung lesions and death of guinea pigs. However, it was found that the intratracheal injection of pneumococcus hemotoxin prepared by the method of Avery and Neill only occasionally produced the characteristic reaction caused by the intratracheal injection of the anaerobic autolysates. From these experiments we believe, therefore, that the necrotizing and lung-toxic principles, and probably the pneumococcus hemotoxin also, are all separate entities in the anaerobically produced autolysates described.

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SURVIVAL OF THE VIRUS OF POLIOMYELITIS FOR EIGHT YEARS IN GLYCEROL.

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The occurrence of epidemics of poliomyelitis raises the question of the length of time which the etiological agent or virus of the disease will survive outside of the animal body. The power of this virus to resist adverse conditions is of interest not only because of its relation to the natural history of the disease, but also in view of the possible application of such information to experimental methods. It was therefore considered worth while to record an instance of the survival of poliomyelitis virus for 8 years in glycerol, particularly since the infectivity of the material remained almost unchanged during this period.

The mechanism responsible for the epidemic nature of the disease has aroused considerable speculation. Flexner and Amoss (1) have pointed out the great variation in the infecting power of a strain of virus much used in experimental work. They (2) suggested that the ability of the virus to survive a long time in mild antiseptics might indicate that a recrudescence of poliomyelitis in a given locality was due to renewed activity of virus which had been dormant since a previous epidemic.

Many attempts have been made to immunize monkeys against poliomyelitis by the use of attenuated virus. The methods used to decrease the infectivity of the material, while preserving its antigenic power, have usually been based on the use of chemical or physical agents, such as phenol, glycerol, heat, and drying. The results have almost always been disappointing, due to the fact that a certain number of animals contracted the disease during the treatment designed to immunize them against it. Such failures were probably caused by the extraordinary resistance shown by some samples of

virus. The persistence of infectivity after long exposure to glycerol is an indication of the hazard of attempts to immunize human beings by the use of virus attenuated by the ordinary methods.

The possibility of exposing poliomyelitis virus to mild antiseptics over long periods is important also from its application to the views of those investigators who consider that the disease is due to a streptococcus. Although Long (3) has cultivated streptococci from pieces of brain tissue which had been kept in 50 per cent glycerol for 303 days, it seems improbable that the organisms could survive in the chemical for a period of 8 years. Needless to say, the material used by us was cultured and streptococci proved absent before it was inoculated into animals.

The first report of the survival of poliomyelitis virus in glycerol is that of Flexner and Lewis (4), published in 1910. These investigators kept the material in 50 per cent glycerol for 7 days and then found it infectious for a monkey. In the same year, Römer and Joseph (5) stated that they had produced poliomyelitis in monkeys with material which had been kept in 100 per cent glycerol for 5 months. 4 years later, in 1914, Flexner, Clark, and Amoss (6) described an experiment in which spinal cord was kept 25 months in 50 per cent glycerol, after which it produced the disease when inoculated in a monkey. Flexner and Amoss (7), in 1917, recorded the successful transfer of virus which had been in glycerol for 6 years. In this case, repeated inoculations of large doses were required to infect the animal, and the authors felt that there had been a definite decline in virulence of the virus.

In the course of investigations on poliomyelitis at The Rockefeller Institute, specimens of the central nervous systems of monkeys which succumb to the disease are removed under rigid aseptic precautions, cut in pieces about 1 cm. in diameter, and placed in a considerable volume of 50 per cent glycerol. Tightly stoppered glass bottles are used and the stopper covered with tin-foil to prevent evaporation. The specimens are kept in the refrigerator at a temperature of approximately 4°C. The glycerol used is free from oxalates and sulfates.

The material employed in the following experiment was a mixture of two strains (M. A. and K.) which had been used for a long time to produce experimental poliomyelitis in monkeys (8). The animals from which the virus was obtained were prostrate on or before the 7th day after inoculation. Spinal cords from seven different monkeys were pooled before grinding and inoculating.

Experiment 1.—October 31. Monkey (*Macacus rhesus*) inoculated intracerebrally¹ with 1.5 cc. of a 20 per cent suspension of pooled 1920 mixed virus. November 8, excitement, tremor, and well marked ataxia were noted, with a tendency to fall when jumping about the cage. There was weakness of arms and legs, with inability to raise the arms above the head. November 9, the animal was prostrate, unable to move either arms or legs, and the respiration was barely perceptible. The monkey was killed by ether and the central nervous system removed under aseptic precautions.

Macroscopic examination: The cord was rather soft and edematous. Tiny, punctate, hemorrhagic areas were scattered through the cord and medulla.

Microscopic examination: The meninges showed a slight infiltration with lymphocytes and endothelial cells. Perivascular infiltration with lymphocytes was noted in the cord and medulla. Various degrees of degeneration of nerve cells of the cord were seen, particularly marked in the anterior horns. Here many examples of active neuronophagia were found, and in some places a polymorphonuclear reaction was present around necrotic nerve cells. Some increase in glia elements was noted. The intervertebral ganglia showed diffuse lymphocytic infiltration and some nerve cell degeneration.

Experiment 2.—November 9. Monkey (*Macacus rhesus*) was inoculated intracerebrally with 1 cc. of a fresh 5 per cent suspension of spinal cord and medulla of the previous monkey. November 12, the animal was slow in its movements about the cage, was somewhat excited, and showed definite ataxia. November 13, weakness of both arms and legs was observed, and the ataxia was more marked. November 14, the condition was slightly more marked. November 15, there was little change. November 16, the animal was much more excited and weaker, and both arms were paralyzed. November 17, the monkey was found prostrate and moribund. It was promptly killed with ether and an autopsy performed.

Macroscopic examination: The cord was somewhat injected and bulged from edema when cut. Scattered, small, soft hemorrhagic areas were found unevenly distributed throughout its length and the medulla.

Microscopic examination: Fairly well marked lymphocytic reaction was present in the meninges. The blood vessels were congested. Perivascular infiltration with lymphocytes was present throughout the cord and medulla. The nerve cells of the cord showed marked degenerative changes, ranging from slight to complete dissolution. A sharp reaction of lymphocytes and mononuclear cells about the degenerated nerve cells was present. The intervertebral ganglia showed diffuse lymphocytic infiltration.

The virus was passed through three more animals in series, with gradually decreasing doses. All the animals were prostrate within 8 days, even when injected with a dose as small as 0.2 cc. of a Berkefeld filtrate of a 5 per cent suspension. The gross and microscopic changes, as well as the symptoms produced, were in all instances characteristic.

¹ All monkey inoculations were carried out under ether anesthesia.

DISCUSSION.

In a paper by Flexner and Amoss, in which the successful transmission of poliomyelitis material kept for 6 years in glycerol was described, appreciable difficulty was experienced in obtaining infection in the first monkeys inoculated. A considerable length of time intervened between the introduction of the nervous tissues and the development of symptoms. In several instances, repeated injection of virus was required to produce the disease. These facts were recorded in order to emphasize the long survival as well as reduction in activity of the virus following the long glycerolation. In view of the results described in this paper, in which the virus has been shown to maintain maximal potency after glycerolation for 8 years, the attenuating action of the chemical may well be questioned. That the specimens of virus should have retained the original high infectivity so that on the first inoculation of the glycerolated tissues the monkeys should have developed typical symptoms and lesions of experimental poliomyelitis is not only an unusual occurrence but perhaps one of epidemiological significance.

CONCLUSIONS.

1. An instance of successful inoculation of poliomyelitis virus after preservation for 8 years in 50 per cent glycerol is reported.

2. The virulence of the material injected remained essentially unchanged during this period.

3. The fact that poliomyelitis virus will survive in glycerol for so great a period may be taken as further indication of the improbability of streptococci as the inciting organisms.

4. Poliomyelitis virus would seem to vary in its resistance to glycerolation.

5. The remarkable persistence of active virus outside of the body may have a bearing on the epidemiology of poliomyelitis.

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